6-1996

Progesterone Regulation of Insulin like-Growth Factor : Binding Protein-5 Gene Transcription in Human Osteoblasts

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ABSTRACT

PROGESTERONE REGULATION OF INSULIN LIKE-GROWTH FACTOR BINDING PROTEIN-5 GENE TRANSCRIPTION IN HUMAN OSTEOBLASTS

by

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Insulin-like growth factor binding protein-5 (IGFBP-5) is produced by osteoblasts and potentiates IGF mitogenic actions in osteoblast cultures. Progesterone (PG) increases osteoblast proliferation and increases osteoblast IGFBP-5 expression. Mechanisms underlying PG induction of IGFBP-5 expression were investigated in U2 human osteosarcoma cells. In nuclear run-on analyses, PG increased IGFBP-5 gene transcription to 400% of control. To define the underlying mechanisms, the human IGFBP-5 gene proximal promoter was cloned and sequenced. The proximal 500 bp of this region contains TATA and CAAT boxes, five putative PG response element (PRE) half-sites and two tandem CACCC boxes. Chloramphenicol acetyltransferase (CAT) reporter constructs containing up to 753 bp of the human IGFBP-5 gene 5'-flanking sequence were made. In U2 cells transfected with reporter construct pCAT753 containing positions -753 to +23, PG increased CAT activity to 134% of untreated control activity ($P<0.01$). Cotransfection with a PG receptor isoform A (PR-A), but not PR-B, expression vector increased PG induction of CAT activity to 200-300% of control (cells with vector alone

and no PG treatment). Deletion analysis of the human IGFBP-5 promoter indicates that PG induction of IGFBP-5 gene transcription does not require the PRE half-sites but does require the region from -162 to -124 containing tandem CACCC box sequences. Mutation of the proximal CACCC box at -139 eliminated PG induction of CAT expression. Gel shift analysis using a -162 to -124 DNA fragment, U2 cell nuclear extracts and purified PR proteins indicates that nuclear factors bind to the proximal CACCC sequence at -139, and that PR-A (but not PR-B) alters the pattern of transcription factor interaction with CACCC sequences. Results suggest that PG may stimulate human IGFBP-5 transcription via a novel mechanism involving the interaction of PR-A and CACCC binding factors.

LOMA LINDA UNIVERSITY

Graduate School

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A Dissertation Submitted in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy in Biochemistry

June 1996

Each person whose signature appears below certifies that this dissertation in their opinion is adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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ACKNOWLEDGMENTS

I would like to thank the members of my guidance committee for their suggestions. I also would like to thank Dr. R. B.Wilcox and Dr. R. Huseby for their encouragement and stimulation of my interest in Endocrinology. I would like to thank Dr. M.R. Drachenberg who first introduced me to molecular biology techniques. I would like to thank the members of the Mineral Metabolism Unit for their support and advice. I would also like to thank Dr. S. Mohan for his encouragement and expert advice. Finally, I like to give special acknowledgments to Dr. T. Linkhart and Dr. D. Strong for their help and guidance throughout the course of my graduate study.

This research was supported in part by grants from Loma Linda University and the Department of Veterans' Affairs.

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ABBREVIATIONS

A	Absorbance or optical density
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
bp	Base pairs
CAT	Chloramphenicol acetyltransferase
cDNA	Complementary DNA
CD-FBS	Charcoal-dextran treated fetal bovine serum
Ci	Curies
cpm	Counts per minute
CS	Calf serum
CTP	Cytidine 5'-triphosphate
dATP	Deoxyadenine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
DEPC	Diethylpyrocarbonate
DMEM	Dulbeccos Modified Eagles Medium
DMSO	Dimethly sulfoxide
DNA	Deoxyribonucleic acid
DTE	Dithioerythritol
DTT	Dithiothreitol

EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
ERE	Estrogen response element
g	Gram
GTP	Guanidine 5'-triphosphate
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
h	Hour
HBC	Human bone cells
HEPES	4-(2-hydroxyethyl)-piperazine ethnesulfonic acid
IAA	Isoamyl alcohol
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
kb	Kilobase
kDa	Kilodalton
KCl	Potassium chloride
min	Minute
$MgCl_2$	Magnasium chloride
MOP	3-N-morpholino propanesulfonic acid
mRNA	Messenger RNA

NaCl	Sodium chloride
NaH_2PO_4	Sodium (monobasic) phosphate
NaOAc	Sodium Acetate
NaOH	Sodium hydroxide
PCR	Polymerase chain reaction
PG	Progesterone
PMSF	Phenylmethane sulfonyl fluoride
PR	Progesterone receptor
PRE	Progesterone response element
RNA	Ribonucleic acid
rpm	Revolution per minute
SDS	Sodium dodecyl sulfate
Tris	Tris (hydroxymethlyl) aminomethane
UTP	Uridine 5'-triphosphate
UTR	Untranslated region
UV	Ultraviolet

CHAPTER ONE

I. INTRODUCTION

A. Bone Metabolism

Bone is not metabolically inert, in fact, it is in a constant state of turnover, in which old bone is being replaced by newbone. The processes of bone turnover and remodeling include bone resorption and formation and occur mainly at the endosteal bone surface (the internal bone surface which has contact with bone marrow) (Jee, 1983). Two types of cells are critical to bone resorption and formation: 1) bone resorbing cells, osteoclasts, which demineralize bone and digest collagen and noncollagen proteins of the bone matrix, and 2) bone forming cells, osteoblasts which synthesize and mineralize the bone matrix.

1. Osteoclast

Osteoclasts are unique and highly specialized cells. They arise from hematopoietic mononuclear cells in the bone marrow (Roodman et al., 1985). The most likely stem cell from which osteoclast is formed is a colony forming cell for the granulocyte-macrophage series (CFU-GM) (Scheven et al., 1983). Osteoclasts are formed by the fusion of precusors on or near the bone surface resulting in large multinuleated cells. Osteoclasts contain lysosomes, nonuniform or pleomorphic mitochondria, and a specific area of the cell membrane adjacent to the bone surface called the ruffled border. The ruffled border is the area of the bone surface which is undergoing resorption. The attachment of osteoclasts to the bone surface requires cell membrane

resorption. The attachment of osteoclasts to the bone surface requires cell membrane bound proteins called intregins. Osteoclasts resorb bone by producing proteolytic enzymes and hydrogen ions under the ruffled boarder area (Blair et al., 1989).

Osteoclastic bone resoption may be stimulated by factors that enhance proliferation of osteoclast progenitors, cause differentiation of osteoclast progenitors to mature cells, or activate of mature mutinuleated cells to resorb bone. Therefore, by inhibiting the agent that blocks proliferation of precursors or inhibiting resorption by the mature multinucleated cells, one could inhibit bone resorption. Factors which have been shown to stimulate osteoclastic bone resorption include parathyroid hormone (PTH) (McSheehy and Chambers, 1986), 1,25-dihydroxyvitamin D (Roodman, et al., 1985), interleukin-1 (IL-1) (Thomson et al., 1986), insulin-like growth factors (IGFs) (Mochizuki et al., 1992), and tumor necrosis factor α (TNFα) (Bertolini et al., 1986). Factors which have been shown to inhibit osteoclastic bone resorption include calcitonin (Chambers and Magnus, 1982), estrogen (Jilda et al., 1992), neutral phosphate (Raisz and Neimann, 1969), γ-intreferon (Gowen et al., 1986), and transforming growth factor (TGF)-ß (Chenu et al., 1988). In addition, bis phosphonates, synthetic non-hydrolyzable pyrophosphate analogs, inhibit bone resorption and are used pharmacologically for this purpose (Kleerekoper and Avioli, 1993).

2. Osteoblasts

Osteoblasts arise from cells in the condensing mesenchyme which become committed as preosteoblasts and then differentiate into mature osteoblasts. During the process of bone formation, some osteoblasts become osteocytes encased in calcified bone. There are several steps required for bone formation which are under the control of the osteoblast : 1) synthesis and intracellular processing of the type I collagen; 2) secretion and extracelluar processing of the collagen; 3) the formation of microfibrils, fibrils, and fibers from the collagen; 4) maturation of the collagen matrix with subsequent nucleation and growth of the hydroxyapatite crystals; 5) synthesis and secretion of non-collagen bone matrix proteins; 6) osteoclast formation and bone resorption (Puzas, 1993).

Since osteoblasts play an important role in bone formation processes, factors that regulate osteoblast functions, such as type I collagen synthesis or osteoblast proliferation, could greatly affect bone formation. Factors which regulate ostoblast functions include: PTH, 1,25 $(OH)_2D_3$, TGF-ß, bone morphogenic proteins (BMPs), insulin-like growth factors (IGFs), glucocoricoids and sex steroids (Canalis, 1993; Puzas, 1993). The effects of IGFs and sex steroids on osteoblasts will be described in the following sections.

3. Bone remodeling

Bone remodeling is a process in which a number of cellular fuctions participate in the coordinated resorption of old bone and formation of new bone. Two phases are essential for bone remodeling (Eriksen, 1986). The first phase is bone resorption which

involves the replication and differentiation of osteoclast progenitors, and migration of differentiated osteoclasts to the bone surface, where they displace lining cells at the site of resorption activation. The mature osteoclast forms a ruffled border area surrounded by the clear or sealing zone, and then releases hydrogen ions to disslove the bone mineral and lysosomal enzymes to degrade the underlying matrix. The second phase of the bone remodeling cycle is the formation phase in which the osteoblasts replace resorbed bone. Two major factors can greatly influence the bone formation phase: a) the presence of resorbed bone surface which serves as a template for bone formation; b) the release of local growth factors from cells or matrix which are required for osteoblast proliferation (Parfitt, 1987). Imbalance in the bone remodeling process can lead to a pathological state. The imbalance in the remodeling process in which an increase in bone resorption is not coupled with an increase in bone formation can lead to osteopenia. An inadequate coupling of bone formation to resorption can result in osteoporosis. In contrast, an increase in bone formation without a balanced increase in bone resorption can cause osteopetrosis which consists of excessive endosteal formation and calcification and leads to spontaneous fractures and occlusion of the marrow space.

B. Osteoporosis and Sex Steroid Hormones

One of the most prevalent pathological states that results from imbalance in the bone remodeling cycle is osteoporosis. Osteoporosis is a disorder in which rates of bone resorption exceed bone formation, resulting in gradual but progressive bone loss and

development of nontraumatic or atraumatic fractures (Kleerekoper and Avioli, 1993). Osteoporotic fractures may affect any part of the skeleton except the skull. The incidence is higher in whites than in blacks, and higher in women than men. Osteoporosis is responsible for about 300,000 hip fractures and more than 1,000,000 total fractures each year in United States. Current estimates indicate that each new case of osteoporotic hip fracture will cost approximately $40,000 and that the annual expenditure for short-term care following osteoporotic hip fractures has already exceeded $8 billion in the United States (Kleerekoper and Avioli, 1993).

Sex steroid hormones are important in maintaining and preserving bone mass. Albright et al. (1940) first noted that osteoporosis was seen in women with oophorectomy before the usual age of menopause and first postulated that ovarian loss was in some way causing the premature development of osteoporosis (Albright et al., 1940). Aitken et al. (1973) showed that women whose ovaries were removed only 3 years previously had a lower bone mass than women of the same age who had their uterus removed but whose ovaries were left in place. Therefore, the loss, or lack of estrogen and other sex steroids produced by the ovary is important in the development of osteoporosis in women (Riggs et al., 1986). The principle effect of estrogen withdrawal is an increase in bone resorption with no change in, or inadequately increase in, bone formation (Arlot et al., 1984). *In vitro* experiments suggest that estrogen indirectly decreases bone resorption by decreasing the synthesis of cytokines, such as interleukin-6, which are present in the bone microenvironment and play a role in the stimulation of bone resorption (Jilka et al.,

1992). Thus, estrogen will slow the rate of bone loss without affecting bone formation. Clinically, the ability of estrogen to increase bone mass is limited, with the best result being a 2-4 % annual increase for 2 years (Kleerekoper and Avioli, 1993).

Postmenopausal women do not only lack estrogen but also lack progesterone (PG). In the normal human mentrual cycle, estrogen and PG secretion are linked. PG secretion is high in the luteal phase of the ovalutory cycle, is low in the follicular phase, and is a key hormone essential for the regulation of reproductive function in the body (Clarke and Sutherland, 1990). Therefore, in the postmenopausal state, menstruation ceases and the production of both estrogen and PG is also halted. Since sex steroid hormones produced from the ovary are important in maintaining and preserving bone mass, it is likely that PG also plays an important role in bone metabolism.

Several studies have shown that treatment of postmenopausal women with progesterone alone reduces the markers of bone resorption (Abdalla et al., 1985; Eriksen, 1986; Linsay et al., 1978; Lobo et al., 1984; Selby et al., 1985). Treatment of postmenopausal women with a combination of PG and estrogen increases serum alkaline phosphatase and osteocalcin, markers of bone formation, to a greater extent than treatment with estrogen alone (Christiansen et al., 1985). Using oophorectomized adult beagle dogs as a model for bone metabolism, Karamobolova et al. (1986) and Snow et al. (1985) demonstrated that PG treatment promoted bone formation. In contrast, estrogen decreased the number of osteoclasts but did not promote bone formation (Karamboloba et

al., 1987). Therefore, it appears that PG exerts its effects on bone formation whereas estrogen exerts its effects on bone resorption to increase bone mass.

Although clinical and animal studies have established that PG prevents bone loss by promoting bone formation, the detailed mechanisms underlying the protective effects of progestin are still unclear. *In vitro* studies, suggest that PG stimulates cell proliferation and differentiation in human osteoblast-like cells (Lau et al., 1994; Scheven et al., 1992; Tremollieres et al., 1992; Verhaar et al., 1994). Human osteoblast-like TE-85 cells were found to express progesterone receptors (PRs) and respond to PG in a dose dependent manner with increased cellular alkaline phosphatase activity (Wei et al., 1993). Since PRs are required for PG to mediate its effects, the presence of PRs in human osteoblasts suggests that PG has direct effects on osteoblast functions. PG appears to increase osteoblast proliferation by modulating the insulin-like growth factor (IGF) system in the osteoblast microenvironment (Lempert et al., 1992; Tremollieres et al., 1992).

C. Molecular Mechanisms of Progesterone Action

PG is synthesized and secreted after ovulation from the collapsed follicle, the corpus leuteum. The hormone then travels via the blood stream to target cells, enter these cells by simple, or facilitated, diffusion, and then binds to PRs. Upon PG binding, the receptor is activated, and can bind effectively to a specific DNA sequence called the

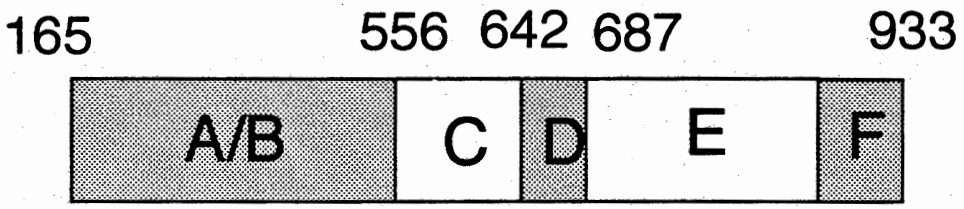
progesterone response element (PRE) to activate gene transcription. A simplified model of PG action is shown in Figure 1.

1. Progesterone receptor and its isoforms

The PR belongs to the steroid hormone receptor superfamily which consists of a large number of genes (Tsai and O'Malley, 1994). It includes receptors for the steroids, estrogen (ER), progesterone (PR), glucocorticoid (GR), mineralocorticoid (MR), and androgen (AR). In addition, it includes receptors for thyroid hormone (TR), vitamin D (VDR), retinoic acid (RAR), and 9-cis retinoic acid (RXR). Amino acid sequence and mutational analyses of these steroid hormone receptors indicate that they can be subdivided into six domains : the A/B domain, DNA binding domain (C domain), hinge region (D domain), ligand binding domain (E domain), and F domain. A schematic representation of the domains is shown in Figure 2. The N-terminal A/B domain is highly variable in length among members of the steroid receptor superfamily. This domain contains a transactivation function (AF) which activates target gene expression presumably by interacting with components of the core transcription machinery, coactivators, or other transactivators (Hollenberg and Evan, 1988; Kumar et al., 1987). The C domain contains two zinc fingers which are responsible for DNA recognition and dimerization (Freeman, 1992). The D domain or hinge region may allow the receptor to bend or alter conformation and often contains a nuclear localization domain (in GR and PR) (Godowski et al., 1988).

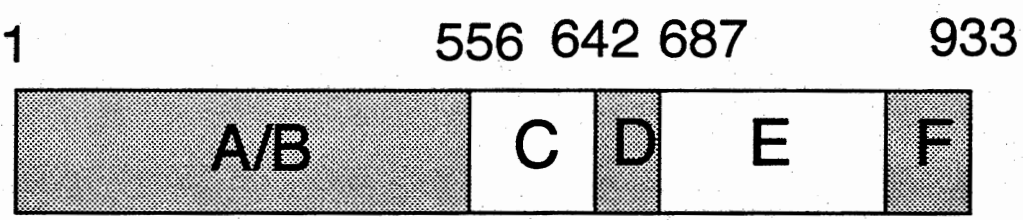
Figure 1. Classical mechanism of PG action. A simplified model of the classical mechanism of PG action. PG enters cells by simple or facilitated diffusion, and binds to intracellular progesterone receptors (PRs). Upon binding PG, the receptors undergo an activation or transformation step. The activated PR can then homodimerize, bind effectively to a specific DNA element, progesterone response elements (PREs), and activate gene transcription.

A



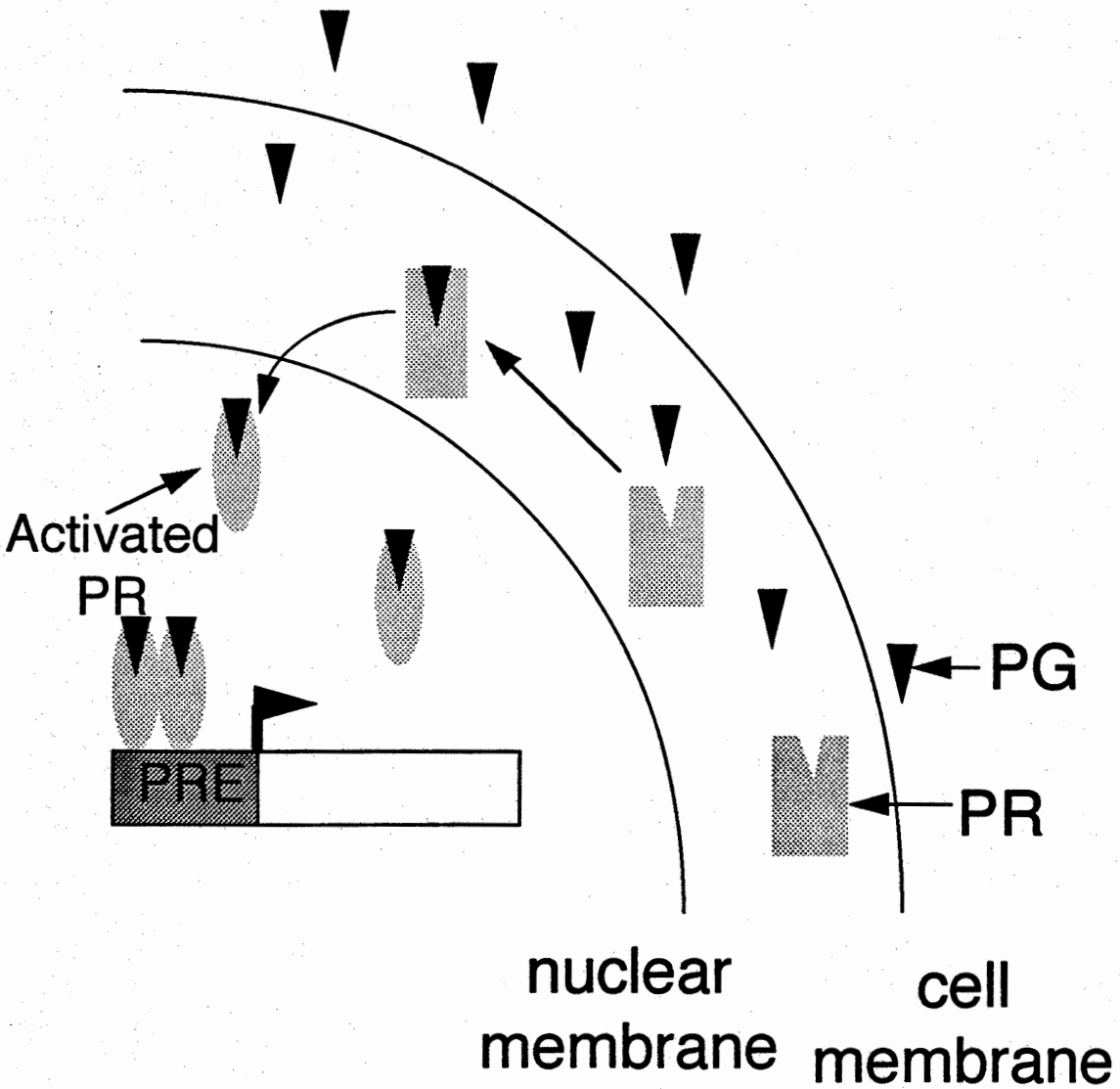
- 94 kDa
- An repressor and activator of transcription

B



- 120 kDa
- An activator of transcription

Figure 2. Progesterone receptor isoforms. Schematic representation of PR isoforms. The modular structure of PR is depicted. A and B represent PR isoform A and B, respectively. Numbers above the receptor represent the amino acid positions of each domain.


Activated
PR
PRE
PG
PR
nuclear
membrane
cell
membrane

The E-region is relatively large compared to other domains. It contains regions important for heat-shock protein association, dimerization, nuclear localization, transactivation, and ligand-binding (Tsai and O'Malley, 1994).In the PR, this region also contains domains essential forintermolecular silencing in which the silencing activity is relieved upon binding to PG (Picard and Yamamoto, 1987). The major dimerization domain of steoid hormone receptors is localized in the C-terminal half of the ligand binding domain (Fawell et al., 1990). This region contains leucine-rich sequences that may form coil-coil interactions as the receptor dimerizes. No specific function has been assigned to the F-domain.

Two forms of PR, amino terminal truncated PR-A and full length PR-B, have been identified in most species with the exception being the rabbit where PR exists as a single unique B-subtype (Loosfelt et al., 1986). In human cells, the PR is expressed primarily as two forms, full length PR-B (M_r 114 kDa) and N-terminal truncated PR-A (M_r 94 kDa) (Kastner et al., 1990). The PR-A isoform lacks the first 164 amino acid at the N-terminus (Figure 2). The two receptors are synthesized from a single gene by alternate initiation of translation from a single PR mRNA (Coneely et al., 1987), or by alternate transcription from two promoters (Kastner, et al., 1990). Since the two receptors in human cells can be individually regulated and transcribed from two distinct promoters, it is likely that the expression levels of PR-A and PR-B can differ with respect to each other in certain target tissues. In support of this hypothesis, it has been shown that the relative expression of PR-A and PR-B in human endometrium changes during the

human menstrual cycle, suggesting that the two different PR promoters can be regulated independently in this organ (Feil et al., 1988). In addition, the relative levels of PR-A and PR-B are different in biopsies of uterine leiomyomas compared with their expression in adjacent normal endometrium (Brandon et al., 1993).

The precise functions of PR-A and PR-B have not yet been well defined. The two isoforms have different functional properties. The transcriptional activities of PR-A and PR-B are dependent upon cell and promoter context (Myer et al., 1992; Vegato et al., 1993). PR-B activates transcription, in most cases, whereas PR-A acts as a trans-dominant repressor of PR-B and other receptors such as GR, MR, and AR (Tung et al., 1993; Vegato, et al., 1993; Wen et al., 1994). The repressor function of PR-A is cell and target gene specific. In a few cases, only PR-A, but not PR-B, activates gene transcription. When an ovalbumin promoter-CAT constructs is cotransfected into Hela cells with a chicken PR-A (cPR-A) or chicken PR-B (cPR-B) expression vector, only cPR-A was able to activate gene transcription (Tora et al., 1988). This is the only example of PR-A specific transactivation of gene transcription. Thus, PR-A has the potential for two functions, activation and repression, depending on the cell and promoter context.

In addition to PR-A and PR-B, two additional transcripts that lack both the A and B translation start site have been mapped. These transcripts contain downstream translation sites that if utilized would generate proteins with a partial DNA-binding domain and complete hormone binding domain (Wei et al., 1990). A third PR isoform

(PR-C) exists in breast cancer cell lines T47D, MCF-7 , and normal human endometrium (Wei and Miner, 1994). PR-C has a molecular weight of approximately 60 kDa and binds specifically to PG and synthetic progestin, R5020, with the same affinity as PR-A and PR-B (Wei and Miner, 1994). The function and regulation of PR-C is still unknown.

2. Activation of the progesterone receptor

PR is activated primarily through binding to PG. PG then transactivates PR to bind to PRE. A series of events takes place once PR binds PG. The physiologically inactive form of the receptor exists as an oligomeric complex with proteins such as heat shock protein (hsp) and immunophilin (Bauleu, 1987; Smith and Toft, 1993). Initially, PR is associated with a multiprotein complex consisting of hsp90, hsp70, and a small protein called p60. The initial multiprotein complex is rapidly displaced by another protein complex consisting of hsp90, p23, and any of the large immunophilins (FKBP52, FKB54, or CyP40) (Smith, 1993; Smith and Toft, 1993). This mature PR complex is thought to be stable unless disrupted by hormone binding. Upon binding to PR, PG induces a conformational change which displaces these multiprotein complexes and allows the uncomplexed receptor to interact with DNA (Tsai and O'Malley, 1994). The hsp containing complexes interfere with, or block, DNA binding (Picard et al., 1988). However, the removal of hsp complexes alone is not enough to activate PR. Ligand binding is essential for activating PR to bind to DNA (Bagchi et al., 1991). The role of

ligand in DNA binding is to induce a conformational change in the ligand binding domain, which exposes the major dimerization domain in this region, allowing receptor dimerization. The dimeric receptor can then bind to a PRE with high affinity (Allan et al., 1992; Tsai et al., 1988)

A major additional process that enhances PG-dependent activation of receptors is phosphorylation. Phosphopeptide-mapping indicates the presence of multiple phosphorylation sites in human PR, predominantly on serine (Sheridan et al., 1989). PR phosphorylation increases after hormone treatment (Sullivan et al., 1988). A significant level of phosphorylation occurs only after the activated receptors bind to DNA (Bachi et al., 1992). Beck et al. (1992) proposed that phosphorylation of human PR involves at least three rounds of phosphorylation: constitutive phosphorylation in the absence of hormone, rapid hormone-dependent phosphorylation, and a much slower phosphorylation after the receptor binds to DNA. Rapid hormone-dependent phosphorylation could be involved in the enhancement of PR-DNA binding or in promoting receptor dissociation from heat shock proteins. The third round of phosphorylation which takes place after PR binding to DNA suggests that this DNA-dependent phosphorylation might be important in regulation of transcriptional activity (Beck, et al., 1992).

Several activators of cell signal transduction pathways can potentiate PG-dependent PR-mediated gene transcription. Effects have been observed with activators of protein kinase A, protein kinase C, and with okadaic acid, a phosphatase inhibitor (Beck, et al., 1992; Beck et al., 1993). These agents enhance the transcriptional

activity of PR but do not effect PR protein synthesis or DNA binding. Unlike ER and chicken PR which can be activated without binding to ligands (Power et al., 1991), ligand-independent activation of human PR has not been successfully demonstrated (Edwards et al., 1995).

3. Glucocorticoid/Progesterone Response Elements (GRE/PRE)

The term hormone response element (HRE) is used for DNA elements which recognize and bind nuclear hormone receptors (Yamamoto, 1985). The first characterized HRE was the glucocorticoid response element (GRE) in the mouse mammary tumor virus (MMTV) promoter. The glucocorticoid-stimulated expression of genes encoding the virus is mediated by short sequences localized upstream of its promoter (Yamamoto, 1985). These DNA sequences confer hormone control to heterologous genes at a distance and in an orientation independent manner (Chandler et al., 1983). This region correlates very well to the long terminal repeat (LTR) of the MMTV promoter and contains as high affinity binding sites for partially purified rat liver GR (Payvar et al., 1983).

After characterization of the MMTV promoter, GRE's of several glucocorticoid inducible genes were described, including human metallothionein IIA (Karin et al., 1983), chicken lysozyme (von der Ahe et al., 1988), moloney murine sarcoma virus (Mikisicek et al., 1986), rat tyrosine aminotransferase (Jantzen et al., 1987), and rat tryptophan oxygenase (Danesch et al., 1983) were reported as glucocorticoid inducible.

Sequence comparison of promoters which bind the glucocorticoid receptor identified a 15 base-GRE consensus sequence of 5'-GGTACANNNTGTTCT-3', where N is any base (Beato et al., 1989). The 3'-half of the consensus sequence contains the motive TGTTCT which is found in almost 90% of the GREs, suggesting that this 3'-half of the GRE plays an important role in interaction with the receptor. The 5'-half of GRE is less conserved among GREs (Beato, et al., 1989).

GR, PR, AR, and MR are related and belong in the same family (Tsai and O'Malley, 1994). These receptors can bind to the same or similar HREs. This may be due to the strong conservation of the DNA binding domain within members of this receptor family. For example, human GR differs from human PR in only 6 of 70 amino acids in the DNA binding domain (Hollenberg et al., 1985; Misrahi et al., 1987). In addition, the target site specificity of the these receptors, especially GR and PR, is very similar (von der Ahe, et al., 1988). In the MMTV promoter, the LTR region can also bind and be activated by PR, AR, and MR, in addition to GR (Arriza et al., 1987; Cato et al., 1987). In the human metallothionein IIA promoter, PR binds to the GRE (Slater et al., 1988).

Although GR and PR can bind to the same, or similar, target sites, these receptors can recognize or use target sites differently (Gowland and Euetti, 1989; Nordeen et al., 1989). Glucocorticoids induce promoter activity to a severalfold greater extent than PG in the MMTV promoter expressed in a human breast carcinoma cell line, T47D (Nordeen, et al., 1989). Mutations of the MMTV promoter differentially affect responses to the two

hormones (Gowland and Euetti, 1989). In addition, there are slight differences in the DNase I footprints generated by the binding of the two receptors to DNA containing the MMTV GRE/PRE (Chalepakis et al., 1988).

Classically, the consensus GRE/PRE is composed of two hexameric half-sites arranged as a palindrome with a 3 bp spacer. Analyses of a series of point substitution and insertion mutations of the distal receptor-binding site of the MMTV promoter have defined optimal recognition sequences for PR and GR (Lieberman et al., 1993; Nordeen et al., 1990). The optimal receptor binding site for GR (GRE) is GGNACAnnnTGTNCC (Nordeen, et al., 1990) and for PR (PRE) is (A/G)GNACAnnnTGTNC(C/T) (Lieberman, et al., 1993). In addition to binding the classical palindromic GRE/PRE, both PR and GR can bind weakly to a hexanucleotide motif, half-site 5'-TGTTCA-3'(Scheidereit et al., 1983). Clusters of this hexanucleotide motif which occur in MMTV, uteroglobin, and other promoters can bind GR/PR and effectively activate transcription through synergistic interaction between the sequences (Bailly et al., 1986; Beato, et al., 1989)

In addition to interacting with GRE/PRE, GR/PR have been shown to interact with other nuclear proteins such as CACCC-box binding proteins (Muller et al., 1991; Schule et al., 1988), c-fos (Jonat et al., 1990), and c-jun (Schule et al., 1990; Yang-Yen et al., 1990) thereby modulating receptor activity. Schule et al. (1988) demonstrated that the cooperatively between GR and CACCC box binding proteins synergized in glucocorticoid induction of gene transcription. In contrast, interaction between GR/PR and c-jun or c-fos resulted in inhibition of GR/PR-induced gene transcription (Schule et

al., 1990; Shemshedini et al., 1991). Overexpression of c-jun prevented the glucocorticoid-induced activation of genes carrying a functional GRE/PRE (Schule et al., 1990). Shemshedini et al. (1991) showed that transcriptional activation by human PR in the human breast cancer cell line, T47D, could be inhibited by increasing intracellular c-fos. Although the precise mechanism of how c-fos and c-jun inhibit hPR/hGR transactivation of gene transcription is still unclear, there is some evidence that c-fos or c-jun might inhibit hPR/hGR activity by competing for factors which are required for activation of the receptors (Shemshedini et al., 1991). Alternatively, Jonat et al. (1990) demonstrated that GR coprecipitated with the AP-1 complex (Fos-Jun) suggesting direct binding of the GR to the AP-1 complex and inhibition of GR-mediated transactivation activity.

D. Insulin-like Growth Factor (IGF) System and Bone Metabolism

1. Components of the skeletal IGF system

The IGF system in bone consists of IGF-I and IGF-II, IGF receptors, IGF binding proteins (IGFBPs) and IGFBP proteases. The IGFs are important autocrine and paracrine regulators of proliferation and differentiation in many cell types including osteoblasts (Jones and Clemmons, 1995; Mohan and Baylink, 1991a; Rosen et al., 1994). IGFs are the most abundant growth factors produced by bone cells and stored in bone (Mohan and Baylink, 1991c). IGFs stimulate two essential components of bone formation, bone cell proliferation (Baylink et al., 1993), and type I procollagen synthesis (Hock et al., 1988),

in vitro. Endogeneous IGFs are potent mitogens for human osteoblasts and contribute to about 40-50% of total basal osteoblast proliferation in serum-free cultures (Mohan et al., 1989). IGFs increase bone formation *in vivo* both in rat and human (Johansson et al., 1992; Zapf and Foresch, 1986). In addition, IGF-I and IGF-II interact synergistically with $1,25(OH)_2D_3$ to increase production of osteocalcin, a marker of bone formation, in rat and human osteoblasts (Fournier et al., 1993). Since there are several components of the IGF system in the bone microenvironment, IGF activities can be regulated by modulating components of the IGF system such as the IGFs, IGF receptors, or IGF binding proteins. Very little is known about the significance and regulation of IGFBP proteases.

2. IGF-I and IGF-II

Historically, IGFs were first discovered in 1957 by Salmon and Daughaday (1957) as a sulfation factor, based on their ability to stimulate cartilage sulfation *in vitro* in the absence of exogenous GH. IGFs were also discovered independently for other biological activities. Foresch et al. (1963) identified the nonsuppressible insulin-like activity of a serum factor that exerted insulin-like action on muscle and adipose tissue even in the presence of anti-insulin antibodies. Dulak and Temin (1973) partially purified polypeptide from serum-free conditioned medium of BRL-3A rat hepatoma cells which stimulated embryo fibroblast proliferation. All three biological factors were unified and termed somatomedin (Daughaday et al., 1972). Later, Rinderknecht and Humbel (1978a;

1987b) purified two distinct somatomedins and called them insulin-like growth factors I and II (IGF-I and IGF-II) based on amino acid sequence homology to insulin.

IGF-I and IGF-II are single chain polypeptides with three intrachain disulfide bridges and molecular weights of 7.6 and 7.5 kDa respectively. IGF-I and IGF-II share 60% amino acid sequence homology (Rinderknecht and Humble, 1978; Rinderknecht and Humble, 1978). The human IGF-I gene is located on chromosome 12 and is approximately 90 kb in length while the human IGF-II gene is approximately 30 kb in length and located on chromosome 11 (Sussenbach, 1989; Tricoli et al., 1984). Two IGF-I mRNA transcripts (IGF-IA and IGF-IB) are expressed during pre- and post-natal development in humans (Han et al., 1988). At least three different size IGF-II mRNA transcripts (4.9, 5.3, and 6.0 kb) resulting from alternative promoter usage and alternate mRNA slicing are expressed in many cell types (Daughaday and Rotwein, 1989; Schneid et al., 1993).

IGFs are important growth factors for pre- and postnatal bone development as demonstrated in homologous *nul* mutation (knock out) transgenic mice. In IGF-I knock-out transgenic mice, surviving mutants have abnormal bone development and are smaller in size (Baker et al., 1993). In IGF-II knock out transgenic mice, new borns homozygous for the disrupted IGF-II gene demonstrate severe growth retardation and die shortly after birth (Liu et al., 1993).

In humans, the highest expression of IGF-I is found in liver (Han, et al., 1988). Liver is also the major source of serum IGF-I, but skeleton IGFs also enter the total

circulating IGF pool. In rats and mice during fetal development, the level of IGF-II in serum is higher than IGF-I. However, during post-natal life the IGF-I levels are higher than IGF-II levels (Daughaday et al., 1982). In constrast to rats and mice, human and guinea pig IGF-II levels in adult serum remain higher than IGF-I levels (Daughaday et al., 1986; Enberg et al., 1984). IGF-I is produced at a much lower level than IGF-II by human osteoblasts *in vitro* (Mohan and Baylink, 1991c). IGF-I production in many human osteoblastic cell preparations is almost undetectable (Mohan et al., 1990). Unlike human bone cells, cultured rat and mouse bone cells produce more IGF-I than IGF-II (Mohan and Baylink, 1991c), suggesting that the regulation of the IGF system in rat and mouse may be different from human.

IGFs in skeletal tissue come from three sites: the circulation, the osteoblasts (Mohan and Baylink, 1991), and the bone marrow stromal cells (Zhang et al., 1991). The osteoclasts do not produce IGFs and IGFBPs. However, IGFs may promote osteoclast differentiation (Mochizuki, et al., 1992). During the bone resorption process, IGFs are released from bone matrix and act on osteoblasts to stimulate osteoblast proliferation. Expression of IGFs is regulated by both systemic and local agents. IGF-I, but not IGF-II, expression is directly regulated by GH (Daughaday et al., 1987). In fetal rat bone cell cultures, PTH increases IGF-I expression at both the protein and mRNA levels (McCarthy et al., 1989). The effect of PTH on IGF-I expression can be duplicated using cAMP analogues (Canalis et al., 1989). In contrast to rat bone cells, PTH, has no effect on the synthesis of either IGF-I or IGF-II in human bone cells (Finkelman et al., 1992).

Pharmacologic doses of cortisol dramatically suppresses production of IGF-I and IGF-II (McCarthy et al., 1989) in cultures of rat osteoblast-enriched cells. Physiological concentrations of $1,25(OH)_2D_3$ (10^{-10}-10^{-7} M) inhibit both basal and PTH stimulated IGF-I but not IGF-II production in mouse calvaria cells (Linkhart and Keffer, 1991). In addition to those systemic factors, several local factors also regulate skeletal IGF synthesis. TGF-ß stimulates the release of both IGF-I and IGF-II in neonatal mouse calvaria (Linkhart and Keffer, 1991), and IGF-I from mouse osteoblasts (Tremolliers et al., 1991). Fibroblast growth factor (FGF) suppresses IGF-I and IGF-II synthesis in osteoblast-enriched cells from fetal rat calvaria (Canalis et al., 1993). Prostaglandin E_2 (PGE_2) stimulates IGF-I production in mouse calvaria cells (Linkhart and MacCharles, 1992) whereas platelet derived growth factor (PDGF) suppresses IGF-I production in rat calvaria cells (Canalis, et al., 1993). BMP-7 stimuates IGF-II production in human bone cells (Knutsen et al., 1995)

IGF production is regulated by sex steroids. Estrogen induces IGF mRNA expression in reproductive tissues (Murphy et al., 1989). In UMR-106 rat osteosarcoma cells, estradiol increases the release of IGF-I and TGF-ß (Gray et al., 1987). Furthermore, the bone forming effect of estradiol is blocked by anti-IGF-I antibodies (Ernst et al., 1989). In addition to estrogen, progesterone (PG) stimulates human bone cell proliferation and increases IGF-II mRNA levels and protein production (Tremollieres et al., 1992).

3. IGF receptors

Two types of IGF receptors have been identified in bone cells: type I receptors and type II IGF/manose 6-phosphate receptors (Mohan and Baylink, 1991c). Most of the cellular effects of IGFs are mediated by binding of the peptides to the IGF receptors and transmitting the signals into the nucleus. The type I IGF receptor is a heterodimeric protein ($\alpha_2\beta_2$) in which the α- (130 kDa) and ß-subunits (90 kDa) are linked by disulfide bonds to form an αß-half-receptor which are subsequently linked to another αß-half-receptor to form the mature $\alpha_2\beta_2$-holoreceptor (Ullrich et al., 1986). Ligand binding specificity is conferred by the cysteine-rich region in the α-subunit extracellular domain (Ullrich, et al., 1986). The human type I IGF receptor gene is located on chromosome 15 (Ullrich, et al., 1986). The signal transduction of the receptor is conferred by a tyrosine kinase activity domain that resides in the cytoplasmic ß-subunit (Czech, 1989). The IGF-I receptor has a high degree of sequence homology (~50-60% sequence identity) to the insulin receptor with the highest homology (84%) in the tyrosine kinase domain (Czech, 1989). The type I receptor has the highest affinity for IGF-I with approximately 100 to 1000 fold lower affinity for insulin and 2 to 15 fold lower affinity for IGF-II (Steele et al., 1988).

The signal transduction of type I IGF receptors is very similar to the insulin receptor. The binding of IGF-I causes tyrosine phosphorylation of an 185 kDa, insulin receptor substrate-1 (IRS-1)(Izumi et al., 1987). The phosphorylated IRS-1 can bind to at least two src homology 2 (SH2)-containing proteins: 1) the p85 regulatory subunits of

phophatidylinositol-3 kinase (PI-3 kinase) (Yamamoto et al., 1992); and 2) growth factor receptor-bound protein 2 (Grb2) (Skolnik et al., 1993). The binding of IRS-1 to the p85 of PI-3 kinase results in the activation PI-3 kinase and phosphorylation of phosphotidyl inositol 4,5-bisphosphate (PIP_2). The subsequent formation of phophatidylinositol-3,4,5 triphosphate serves as a signal for cell growth. The activated Grb2 can further complex with the guanine nucleotide exchange factor, Son of sevenless (Sos) (Skolnik et al., 1993). Formation of this complex is necessary for the activation of Ras (Baltensperger et al., 1993). Activated Ras then activates Raf causing the activation of mitogen activating protein kinases (MAP kinases) which ultimately transmit signals to the nucleus (Blenis, 1993).

The type II IGF receptor is a monomeric receptor that binds to manose-6-phosphate (Man-6-P) residues on lysosomal enzymes (Nielsen, 1992). Type II IGF-II/ Man-6-P receptors bind IGF-II with high affinity and bind IGF-I with a 500 fold lower affinity. This receptor does not bind insulin (Nielsen, 1992). The binding site for IGF-II is distinct from the binding site for Man-6-P and Man-6-P containing glycoproteins (Braulke et al., 1988). In mice, IGF-II/Man-6-P receptor (Igf2r) and IGF-II (Igf-2) genes are subject to parental imprinting such that only the maternal allele of Igf2r and the paternal allele of Igf2 are preferentially expressed (Barlow et al., 1991; DeChiara et al., 1991). In humans, however, the Igf2 gene is imprinted while the Igf2r gene is not (Ohlsson et al., 1993).

The signal transduction pathway of the type II IGF-II/ Man-6-P receptor is not well understood. IGF-II mediates an increase of Ca^{++} influx which, in turn, stimulates DNA synthesis in BALB/c 3T3 cells (Nishimato et al., 1987). The increase of Ca^{++} influx and DNA synthesis can partially be explained by the following pieces of data: 1) the receptors couple to GTP-binding proteins; and 2) binding of IGF-II to the receptor stimulates binding of GTPγS to G_{i2} and increases GTPase activity which results in an increase in Ca^{++} influx (Muruyama et al., 1990), and ultimately transmits the signal into the nucleus.

The physiological role of the IGF-II/Man-6-P receptor is unclear. However, the receptor may function in degradative pathways to remove IGF-II from the extracellular environment (DeChiara et al., 1990). It remains uncertain whether the biological action of IGF-II is mediated via the type II IGF-II/Man-6-P receptor. Several lines of evidence suggest that the biological action of IGF-II may be mediated by the IGF-I receptor. Treating H-35 hepatoma cells (Mottola and Czech, 1984), normal hepatocytes(Hartmann et al., 1992), L6 myoblasts (Kiess et al., 1987), and ovarian granulosa cells (Adashi et al., 1990) with antibodies directed against the IGF-II/Man-6-P receptor has no effect on biological responses to IGF-II. In addition, a blocking monoclonal antibody (αIR-3) against the IGF-I receptor blocked both IGF-I and IGF-II stimulated [^{3}H]-thymidine incorporation into DNA in human fibroblast cultures (Flier et al., 1986; Van Wyk et al., 1985). However, in bone cells, which contain type II IGF-II receptors, blocking antibodies against the type II IGF-II/Man-6-P receptor inhibited IGF-II induced

osteoblast proliferation while the IGF-I blocking antibody, αIR-3, was ineffective in blocking IGF-II stimulated proliferation in mouse osteoblasts (Mohan and Baylink, 1991c), suggesting that the type II IGF-II/ Man-6-P receptor is important for bone cell proliferation. Consistent with this conclusion, treatment of human osteoblasts with PG increases the type II IGF-II/Man-6-P receptor mRNA levels and increases bone cell proliferation (Lempert et al., 1992).

4. IGFBPs

In addition to the IGFs and their receptors, IGFBPs present in the bone cell microenvironment also play an important role in regulating IGF activities. In the circulation and throughout the extracellular space, the IGFs bind with high affinity to IGFBPs. Six IGFBPs of different molecular weights (IGFBP-1, -2, -3, -4, -5, and -6) have been identified. (Shimasaki and Ling, 1991). The IGFBPs share structural homology with each other and specifically bind the IGFs. All human IGFBPs, except IGFBP-6, contain 18 homologous cysteines; twelve of which are located at the N-terminal in the first one-third of the total amino acid sequence (Shimasaki and Ling, 1991). The physiological function of IGFBPs is still unclear. The IGFBPs 1) act as storage and transport proteins for the IGFs, 2) prolong the half-lives of IGFs, 3) provide tissue and cell type-specific localization, and 4) modulate the biological actions of the IGFs (Jones and Clemmons, 1995).

Five different molecular weight IGFBPs (25, 29, 34, 38.5, and 41.5 kDa forms) have been identified in human bone cell (HBC) cultures (Mohan and Baylink, 1991b). Most HBC (except MG63 osteosarcoma cell line) cultures produce IGFBP-2 through -6 (Mohan and Baylink, 1991b). No immunoreactive IGFBP-1 is detected in HBC cultures (Mohan and Baylink, 1991b). Under serum free culture conditions, the 25 kDa and 38.5-41.5 kDa IGFBPs are the two most abundant IGFBPs produced by bone cells (Mohan and Baylink, 1991b). The 25 kDa protein is IGFBP-4; the 38.5-41.5 kDa protein is IGFBP-3; and the 34 kDa protein is IGFBP-6. A 24 kDa protein detected in some HBC cultures is a proteolytic fragment of IGFBP-5 (Bautista et al., 1991; Mohan and Baylink, 1991b). The amount and type of IGFBPs produced by normal HBC derived from different sites and by different osteosarcoma cell lines are different (Hassager et al., 1992; Mohan and Baylink, 1991b), suggesting that the pattern of IGFBP expression is cell type and differentiation stage specific. In addition, expression of each of the IGFBPs produced by HBCs is modulated differently by various effectors (Hassager, et al., 1992). IGFBPs in HBCs are regulated by various systemic and local factors. The regulation of each IGFBP, especially in osteoblastic cells, is described below.

a. IGFBP-1

Human IGFBP-1 is a 25 kDa protein produced by human hepatoma cells, Hep G2 (Rechler and Nissley, 1990), and rat osteosarcoma cells, UMR-106 (Koutsilieris and Polychronakos, 1992). In MG63 human osteosarcoma cells, Campell and Novak (1991)

found that IGFBP-1 inhibited IGF-I induced cell proliferation. However, IGFBP-1 has no effect on IGF-I induced cell proliferation in chick calvaria cells (Mohan et al., 1989). Since IGFBP-1 is produced by a limited number of HBCs and human osteosarcoma cell lines, it is not likely that IGFBP-1 plays an important role in modulating the IGF system in the bone microenvironment.

b. IGFBP-2

Human IGFBP-2 is a 31.4 kDa protein (Lamson et al., 1989) which binds to IGF-II with 10 fold higher affinity than it binds to IGF-I (Brinkert et al., 1989). IGFBP-2 is expressed in various cell types including adult human brain and liver cells (Brinkert, et al., 1989), adult rat brain, testes, and ovary (Margot et al., 1989), and rat osteoblasts (Schmid et al., 1989; Schmid et al., 1992). However, in human bone cells, IGFBP-2 is expressed only in the osteosarcoma, TE85, and not in other human osteoblast-like cells (Bautista, et al., 1991; Mohan and Baylink, 1991). At high IGFBP-2: IGF-I ratio (10:1), IGFBP-2 inhibits IGF-I induced rat calvaria cell proliferation (Fayen et al., 1991). In rat osteoblasts, IGFBP-2 expression is increased by $1,25(OH)_2D_3$, triiodothyronine (T_3), and PTH, but does not respond to GH or retinoic acid (Chen et al., 1991; Schmid, et al., 1992).

c. IGFBP-3

Human IGFBP-3, the main carrier of IGFs in serum, is a 46-53 kDa protein that contains three potential N-linked glycosylation sites (Baxter and Martin, 1989). In serum, most of the IGF circulates as a 150 kDa complex consisting of IGF-I or IGF-II, IGFBP-3 and an 85 kDa acid-labile subunit (ALS) (Baxter and Martin, 1989). ALS contains several leucine rich domains which interact with IGFBP-3 (Leong et al., 1992). The binding of IGFs to IGFBP-3 in the presence of ALS stabilizes IGF binding and prolongs the half-lives of IGFs from less than 10 min for free IGFs to 12-15 h for IGFs in 150 kDa ternary complexes (Guler et al., 1989; Hodgkinson et al., 1989). IGFBP-3 is produced in many peripherral tissues, but not liver, while ALS production is observed mostly in liver (Rechler, 1993). IGFBP-3 may enhance or inhibit the actions of IGFs depending upon cell types and culture conditions. When IGFBP-3 is added at the same time with IGF-I, IGFBP-3 inhibits basal, and IGF induced, bone cell proliferation. However, if the cells are preincubated with IGFBP-3 before adding IGF-I, IGFBP-3 enhances IGF actions at low concentration but inhibits IGF actions at high concentration (Mohan, 1993).

IGFBP-3 expression is regulated by various local and systemic regulators. IGF-I and GH stimulate IGFBP-3 production *in vivo* (Schmid et al., 1989; Zapf et al., 1989). IGF-I , TGF-ß, GH, $1,25(OH)_2D_3$, and bone morphogenic protein (BMP)-7 stimulate while dexamethasone inhibits IGFBP-3 production in human osteoblast-like cells (Knutsen et al., 1995; Nakao et al., 1994; Scharla et al., 1993; Schmid et al., 1989).

d. IGFBP-4

Human IGFBP-4 is a 24 kDa binding protein with N-linked glycosylation sites which inhibits IGF actions in all cell types and culture conditions tested (La Tour et al., 1990; Mohan, et al., 1989). IGFBP-4 accounts for almost 90% of all IGFBPs secreted from mouse osteoblasts (Scharla et al., 1991). Addition of exogenous IGFBP-4 to mouse osteoblasts, MC3T3-E1, inhibited basal and IGF-I induced cell proliferation (Amarnani et al., 1993). Consistent with these findings, Malpe et al. (1992) reported that addition of IGFBP-4 antisense oligodeoxyribonucleotide to inhibit IGFBP-4 expression stimulated bone cell proliferation In addition to inhibiting bone cell proliferation, IGFBP-4 may play a role in bone cell differentiation. The addition of the potent bone cell differentiation agent, 1,25 $(OH)_2D_3$, increases IGFBP-4 production and decreases bone cell proliferation (Scharla et al., 1993). These results suggest that IGFBP-4 inhibits the proliferative affects of IGFs and allows the cells to start the differentiation processes.

IGFBP-4 expression is increased by PTH, cyclic AMP analogues, and 1,25 $(OH)_2D_3$ (La Tour, et al., 1990; Scharla et al., 1993) and is inhibited by IGF-I, IGF-II, progesterone, and BMP-7 (Knutsen et al., 1995; Lempert et al., 1992; Scharla et al., 1991). Circulating levels of IGFBP-4 may reflect changes in the IGF system in the bone cell microenvironment. Elderly women with hip fractures have high serum levels of PTH and IGFBP-4 (Rosen et al., 1992). In addition, psoriasis patients who have chronic exposure to 1,25 $(OH)_2D_3$ have elevated levels of serum IGFBP-4 (Scharla et al., 1993), consistent with the *in vitro* findings that 1,25 $(OH)_2D_3$ stimulates IGFBP-4.

e. IGFBP-5

Human IGFBP-5 has the molecular weight of 29 kDa and has been purified from human bone (Bautista, et al., 1991), adult rat serum (Shimasaki et al., 1991), cerebrospinal fluid (Binoux et al., 1991), and gliobalstoma T98G cells (Camacho et al., 1992). IGFBP-5 binds with higher affinity to IGF-II than IGF-I (Martin et al., 1990). Human IGFBP-5 is composed of 252 amino acids, contains 18 conserved cysteine residues with O-glycosylation sites (Bautista, et al., 1991; Shimasaki et al., 1991). IGFBP-5 cDNA clones were isolated from human placenta (Shimasaki et al., 1991), U2 osteosarcoma (Kiefer et al., 1991), and rat ovary cDNA (Shimasaki et al., 1991). Of the six IGFBP cDNAs, IGFBP-5 is the most conserved with 97% identity among the human, rat, and mouse amino acid sequences (James et al., 1993). The human and rat IGFBP-5 genes encode multiple mRNA transcripts between 5 and 6 kb (Shimasaki et al., 1991). Normal human osteoblasts express a single 6.0 kb mRNA (Chevalley et al., 1996). IGFBP-5 is expressed in most tissues including testis, uterus, intestine, adrenal, stomach, spleen, heart, lung, muscle, brain, and liver (Shimasaki et al., 1991). The human IGFBP-5 (hIGFBP-5) gene has been cloned and localized to the 2q33-34 region on chromosome 2 tightly linked with, and in the opposite transcriptional orientation as, the human IGFBP-2 gene (Allander et al., 1994). The gene is divided into four exons which span ~33 kilobase (kb) of DNA with the first intron spanning approximately 25 kb (Allander, et al., 1994).

hIGFBP-5 is the major IGFBP stored in bone and has consistently been shown to enhance the mitogenic action of IGFs. Coincubation of mouse MC3T3-E1 osteoblasts with equimolar concentration of IGF-II and IGFBP-5 results in potentiation of IGF-II action (Bautista, et al., 1991). Andress et al. reported that a mixture of IGFBP-5 and IGFBP-6 recovered from an IGF affinity column stimulated IGF-I potentiated DNA synthesis in osteoblasts (Andress and Birnbaum, 1991). Recombinant human IGFBP-5 purified from yeast inhibited DNA synthesis in SaOS2/B10 human osteosarcoma cells (Keifer et al., 1992). However, subsequent studies from the same laboratory showed that recombinant IGFBP-5 stimulated DNA synthesis in osteoblasts (Andress et al., 1993). In addition to enhancing the mitogenic action of IGFs, IGFBP-5 binds with high affinity to hydroxyapatite (Bautista et al., 1991) and to various matrix proteins such as type III and IV collagen, laminin, and fibronectin (Jones et al., 1993). These unique properties of IGFBP-5 suggest that IGFBP-5 plays an important role in fixing IGFs to the extracellular matrix including mineralized bone. Consistent with this observation, recent studies have shown that the skeletal content of IGFBP-5 and serum IGFBP-5 levels decreased by about 32% between the age of 20 and 60 (Mohan et al., 1995; Nicolas et al., 1995), suggesting that these age-related losses of IGFBP-5 may, in part, contribute to age related bone loss.

The storage of the purified 29 kDa IGFBP-5 protein at 4 C overnight leads to the disappearance of the 29 kDa IGFBP-5 band on polyacrylamide gels and appearance of a major band at 24 kDa with lower molecular weight minor bands (Bautista et al., 1991),

suggesting that the 29 kDa IGFBP-5 is degraded by proteases present in the IGFBP-5 preparation. Proteases with molecular weights of 52-72 and 97 kDa which degraded IGFBP-5 in MC3T3-E1 mouse osteoblast cultures have been characterized (Thrailkill et al., 1995). The 52 and 69-72 kDa proteases are cation dependent, inhibited by a tissue inhibitor of metalloproteinase 1, and immunoprecipitated with antisera to human matrix metalloproteinase (MMP)-1 and MMP-2, respectively, suggesting that the murine MMP homolog degrades IGFBP-5 (Thrailkill et al., 1995). The identity of the 97 kDa proteases is still unknown. The production of IGFBP-5 proteases in human osteoblast cultures has recently been demonstrated. IGFBP-4 and IGFBP-5 protease activities are detected in the conditioned medium of U2 human osteosarcoma cells and untransfromed HBCs derived from skull (Kansaki et al., 1994). The proteases have molecular weights of 160 and 67 kDa and are inhibited by aprotinin, zinc chloride, and EDTA (Kansaki et al., 1994). Human fibroblasts also secrete IGFBP-5 proteases (Nam et al., 1994). This protease is calcium-dependent serine protease with properties similar to kallikreins, a family of serine proteases that degrade epidermal and nerve growth factors. The protease is specific for IGFBP-5 and its proteolytic activity is minimally affected by exogenous IGF-II (Nam et al., 1994).

The mechanism by which IGFBP-5 potentiates IGF actions is not well understood. Intact IGFBP-5 adheres to fibroblast extracellular matrix (ECM) (Jones et al., 1993). When IGFBP-5 is associated with ECM, it potentiates IGF-I induced cell proliferation by 100% (Jones et al., 1993) whereas the 21 kDa proteolytic fragment does

not associate with ECM and does potentiate IGF action. Since IGFBP-5 affinity for IGF-I is lowered by approximately 7 fold when associated with ECM, Jones et al. (1993) suggested that IGFBP-5 potentiates IGF actions by fixing IGFs to the ECM with low affinity and thereby making the IGF readily available to IGF receptors. Intact IGFBP-5 is more potent than the proteolytic fragments in potentiation of IGF actions in bone cells and binds directly to bone cells (Mohan et al., 1995). The IGFBP-5 potentiation of IGF action does not involve increased IGF binding to IGF receptors. IGFBP-5 binding to bone cells is not affected by excess of either IGF-I and IGF-II suggesting that IGFBP-5 binds to sites independent of the IGF-receptor (Mohan, et al., 1995).

IGFBP-5 production is regulated by local and systemic factors. In UMR-106-1 rat osteosarcoma cells, IGFBP-5 production is increased by IGF-I, PTH, and cAMP analogs (Conover et al., 1993). In contrast, in U2 human osteosarcoma cells, PTH has no effect on IGFBP-5 production; however, IGF-I increases IGFBP-5 accumulation in conditioned medium by 10 fold compared to control, partly by inhibiting the degradation of intact IGFBP-5 (Connover and Kiefer, 1993). In human osteoblast-like cell cultures, IGFBP-5 production is increased by bone morphogenic (BMP)-7, IGF-I, and IGF-II by pretranslational mechanisms involving increased IGFBP-5 mRNA levels and by posttranslational mechanisms involving decreased IGFBP-5 degradation (Kansaki, et al., 1994; Knutsen et al., 1995). Retinoic acid and glucocorticoid decrease whereas progesterone increases IGFBP-5 production (Chevalley et al., 1996; Lempert et al., 1992; Zhou et al., 1996).

f. IGFBP-6

Human IGFBP-6 has a molecular weigh of 34 kDa with O-linked glycosylation sites (Bach et al., 1992; Zarf et al., 1990). Human IGFBP-6 lacks two of the 18 conserved cysteins found in IGFBPs 1-5 (Shimasaki and Ling, 1991). The distinct property of the IGFBP-6 is that it binds to IGF-II with 20 to 100 fold higher affinity than to IGF-I (Martin et al., 1990), suggesting that IGFBP-6 plays an important role in modulating IGF-II actions. IGFBP-6 inhibits IGF-II stimulated DNA synthesis and glycogen synthesis, but only minimally inhibits IGF-I stimulation (Keifer et al., 1992). Consistent with this observation, Srinivansan et al. (1996) reported that IGFBP-6 inhibited IGF-II induced cell proliferation in MC3T3-E1, mouse osteoblasts. Like other IGFBPs, IGFBP-6 expression is regulated by both local and systemic agents. In human fibroblasts, IGFBP-6 is increased by TGF-ß and agents that increase intracellular cAMP concentration such as dibutyryl cAMP, forskolin, and cholera toxin (Martin et al., 1994). In rat ovary, IGFBP-6 production is stimulated by a combination of FSH and LH (Rohan et al., 1993). The expression of IGFBP-6 in human osteoblasts is increased 10 fold by retinoic acid. In addition, when human osteoblasts are treated with the combination of retinoic acid and 1,25 $(OH)_2D_3$, the IGFBP-6 expression is significantly increased compared to treatment with retinoic acid alone (Zhou et al., 1996).

5. Effect of progesterone on the IGF system in human osteoblasts

PG has been shown to increase bone formation *in vivo* (Scheven et al., 1992) and increase human osteoblast proliferation (Lau et al., 1994; Tremollieres et al., 1992). One of the mechanisms by which PG increases human osteoblast proliferation is through modulation of the IGF system. PG causes an increase in IGF-II expression and induces a transient increase in type I and type II IGF receptor mRNA levels in human osteoblasts (Lempert et al., 1992). Pretreatment of MG63 human osteosarcoma cells with PG increases the sensitivity of the cells to IGF-II stimulation of cell proliferation (Lempert et al., 1992), suggesting that pretreatment with PG alters components of the IGF system and make the cells more responsive to IGF-II. In addition to increasing IGF-II, type I, and type II IGF receptors, PG also decreases the expression of an inhibitory IGFBP, IGFBP-4, and increases the expression of the stimulatory IGFBP, IGFBP-5 (Lempert et al., 1992).

E. Rationale

Although we have previously established that PG increases IGFBP-5 production and mRNA levels, the molecular mechanism by which PG increased IGFBP-5 mRNA levels is still unknown. IGFBP-5 is the most abundant IGFBP stored in bone and is the only IGFBP that binds avidly to hydroxyapatite,thus functioning to fix IGFs into bone. Since IGFBP-5 is an important regulator of the IGF system in bone and its production is affected by PG, it is important to understand the molecular mechanisms underlying PG

induction of IGFBP-5 production. Understanding these molecular mechanisms may help provide a basis for understanding the mechanisms by which other regulators affect IGFBP-5 production in human osteoblasts.

Current FDA approved treatment for osteoporosis, such as estrogen replacement and calcitonin therapies, act by inhibiting the bone resorption component of bone remodeling with very minimal effects on the bone formation component. With current therapies, the net gain in bone mass in osteoporosis patients is limited. As a result, there are few studies showing that current treatments of patients with established osteoporosis reduce the occurrence of new fractures (Kleerekoper and Avioli, 1993). There is a need to develop new therapeutic approaches which can effectively increase bone formation and thus reduce the occurrence of new fractures in osteoporosis patients. Since the IGF system plays an important role in osteoblast proliferation and IGFBP-5 enhances IGF-mediated stimulation of human osteoblast proliferation, a complete understanding of IGFBP-5 regulation in bone will provide us with an important tool to manipulate IGF actions in the bone microenvironment. The findings resulting from this study will be helpful in developing new therapeutic approaches for osteoporosis which will not only preserve, but also increase, bone mass and reduce the occurrence of new fractures.

F. Hypothesis

The mechanism by which progesterone increases IGFBP-5 mRNA levels in human osteoblasts is transcriptional and involves the direct interaction of the activated

progesterone receptor and specific response element(s) in the human IGFBP-5 gene promoter.

G. Specific Aims

The specific aims that were used to guide the research described in this thesis are:

1. To determine the time course effects of progesterone treatment on IGFBP-5 mRNA levels;

2. To determine whether progesterone affects IGFBP-5 mRNA expression primarily through a transcriptional mechanism;

3. To isolate genomic clones containing regulatory elements for the IGFBP-5 gene and identify the promoter region and transcriptional start sites;

4. To identify cis-acting elements in the human IGFBP-5 gene promoter and transcription factors interacting with elements which mediate transcriptional induction of IGFBP-5 gene expression;

5. To identify the PR receptor isoform required for PG-mediated transactivation of IGFBP-5 gene in human osteoblasts.

CHAPTER TWO

II. MATERIALS AND METHODS

A. Materials

Progesterone acetate was obtained from Sigma Chemical (St. Louis, MO) and R5020, promegestone, from Dupont NEN Research Products (Boston, MA). Human osteogenic sarcoma U-2 OS (U2) cells (HTB96) were obtained from the American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM) was obtained from Mediatech, Inc. (Herndon, VA). Fe-supplemented bovine calf serum (CS) and charcoal-dextran treated fetal bovine serum (CD-FBS) were from Hyclone (Logan, UT). Penicillin, streptomycin and trypsin were from Gibco (Grand Island, NY). Crystallized Bovine serum albumin (BSA) and guanidine isothiocyanate were from Fluka (Ronkonkoma, NH). Cell culture plastic ware was obtained from Corning Incorporated (Corning, NY).

Recombinant human IGFBP-5 cDNA (BP5/5) was the generous gift of Drs. C. Dony and K. Lang of Boehringer Mannheim Therapeutics, Penzberg, Germany. This cDNA was a PCR product spanning the entire IGFBP-5 coding region and was cloned into a pUC plasmid. pMSG-CAT was obtained from Pharmacia Biotech (Piscataway, NJ). pJFCAT1 was kindly provided by Dr. J Fridovich-Keil, Dana-Farber Cancer Institute (Boston, MA). pGEM-3Zf(-) and pGEM-7Zf(-) were obtained from Promega (Madison, WI). Human ß-actin cDNA was purchased from the American Type Culture

Collection (Rockville, MD). pCMV-βgal was a generous gift from California Biotechnology Inc. (San Diego, CA)

The human genomic cosmid library was obtained from Clontech (Palo Alto, CA). Magnagraph and NitroPure membranes were obtained from MSI (Westboro, MA). Hybond-N membranes were obtained from Amersham (Arlington Heights, IL). INVαF' competent bacterial cells and the pCRII plasmid were obtained from Invitrogen (San Diego, CA). Low melting point agarose was obtained from FMC Bioproducts (Rockland, ME). Oligodeoxynucleotides were synthesized by Integrated DNA Technologies (Coralville, IO) and by the Center for Gene Therapy, Loma Linda University (Loma Linda, CA). LipofectAMINE was obtained from GIBCO-BRL (Gaithergburg, MD).

The Klenow fragment of DNA polymerase I was from Promega (Madison, WI). Vent (*exo*$^-$) was from New England Biolab Inc. (Beverly, MA). T4 polynucleotide kinase was from Promega (Madison, WI). Restriction enzymes and buffers were from Promega, New England Biolab Inc., and United States Biochemical Corporation (Cleveland, OH). dNTPs, dN_6(hexanucleotide), and poly (dI-dC) were obtained from Pharmacia (Piscataway, NJ). NACS columns were obtained from GIBCO/BRL (Gaithersburg, MD). Mermaid kits were purchased from BIO 101 Inc. (La Jolla, CA). Wizard Miniprep, Wizard PCR DNA purification kits, and reporter lysis buffer were obtained from Promega. Qiagen plasmid Maxi kits were obtained from Qaigen Inc. (Chatsworth, CA). Culture dishes and polypropylene tubes were obtained from Corning Glass Works (Corning, NY). Reporter Lysis buffer was obtained from Promega

(Madison, WI). D-threo-1,2-[^{14}C] Chloramphenicol, α-[^{32} P] dCTP, and γ-[^{32}P] ATP were purchased from ICN Biochemical, Inc. (Irvine, CA). α-[^{32}P] UTP was purchased from Dupont NEN Research Products (Boston, MA). Aprotinin and PMSF were obtained from Sigma Chemial Company (St. Louis, MO). Leupeptin and pepstatin were obtained from Calbiochem-Novabiochem International (San Diego, CA). PR mammalian expression vectors phPR-A and phPR-B (Vegato et al., 1993) were provided by Dr. B. O'Malley, Baylor College of Medicine, Houston, TX. Purified PR-A and PR-B and a specific monoclonal antibody (AB52) which recognizes PR-A and PR-B (Onate et al., 1994) were provided by Dr. D.P. Edwards, University of Colorado Health Sciences Center (Denver, CO). All other molecular biology grade chemicals were obtained from United States Biochemical Corporation (Cleveland, OH), Sigma Chemical Company (St. Louis, MO), and Cal Biochem (LaJolla, CA).

B. Methods

1. Cell culture

U2 cells were maintained in DMEM supplemented with 10% calf serum (CS) at 37 C in a humidified atmosphere composed of 95% air and 5% CO_2. In some experiments, cells were grown 3 days in phenol-red free DMEM with 5% charcoal-dextran treated fetal bovine serum (CD-FBS), transfected, and tested 48 h further in phenol-red free DMEM with 2% CD-FBS. PG was dissolved in ethanol at 10^{-2} M and was further diluted with serum-free DMEM containing 0.1% BSA before adding to the

cells. Control cultures were treated with ethanol diluted to the same extent as for PG treatment.

2. RNA isolation

U2 cells were plated at 5 x 10^5 per 100 mm^2 culture dish in DMEM containing 10% CS and incubated for 24 h. The media was then replaced with serum free media containing 0.1% BSA and incubated for 24 h. Prior to addition of PG, cells were changed to serum free medium containing 0.1% BSA. Cells were treated with 10 nM PG for 0.5 to 24 h and total RNA was extracted using a single-step guanidinium-isothiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). All glassware and water were pretreated with diethylpyrocarbonate (DEPC) to destroy ribonucleases. Solutions were made using pretreated DEPC water and then autoclaved.

For isolation of total RNA from cultured cells, media was removed and cells were harvested by overlaying 1 ml of GIT extraction solution per 100 mm^2 dish. Cell extract was collected in a 50-ml polypropylene tube (Corning Incorporated, Corning, NY) and immediately stored at -80 C. For phenol chloroform extraction the following solution were added sequentially to the cell extract, with complete mixing between each addition: 0.1 volume of 2 M sodium acetate (pH 4.0), 1 volume of DEPC water-saturated phenol and 0.2 volume of DEPC water saturated-chloroform-IAA (49:1). The mixture was then vortexed vigorously, incubated on ice for 15 min, and centrifuged at 10,000 x g for 20 min at 4 C. The aqueous layer was transferred to a new tube, mixed with an equal

volume of isopropanol to precipitate RNA, and stored at -20 C overnight. The RNA pellet was collected by centrifugation at 10,000 x g for 20 min at 4 C. All residual isopropanol was removed and the pellet was washed twice with -20 C 70% ethanol and centrifuged at 10,000 x g for 10 min at 4 C after each washing step. All residual ethanol was removed and the RNA pellet was air dried at room temperature for 10 min and resuspended in approximately 50 µl of DEPC water. The amount of RNA obtained was determined by A_{260}. A_{260}/A_{280} ratios of RNA obtained from this method were greater than 1.8, indicating negligible protein contamination. 100 µg of U2 RNA was routinely obtained from five 100 mm^2 culture dishes of 80% confluent U2 cells. All resuspended RNA was kept at -80 C.

3. Northern Blot Analysis

Total RNA was subjected to Northern blot analysis as described (Scharla et al., 1993). Twenty-five µg of total RNA in 10 µl of DEPC water was mixed with 10 µl of Northern loading buffer. The mixture was incubated at 65 C for 5 min to denature the RNA, and immediately kept on ice until loading. The RNA was fractionated on a 1.5% agarose gel containing 6.6% formaldehyde and 1 x MOPS. Electrophoresis was performed using 1 X MOPS as running buffer. When the xylene cyanol dye front was near the end of the gel, electrophoresis was stopped. The gel was then transferred with 10 X SSC overnight by capillary force onto a Magnagraph membrane. RNA was fixed to the membrane by UV cross-linking (1.6 KJ/m^2) followed by baking at 80 C for 20 min.

The membrane was prewetted with 1 X SSPE , stained with methylene blue solution and washed with heated DEPC water. The stained 28S (5.1 kb) and 18S (1.9) kb ribosomal RNA bands were used to estimate RNA transcript size and to quantitate RNA loading and transfer. Before hybridization, the membrane was destained with 0.2 x SSPE/1% SDS. The RNA blots were prehybridized at 42 C for 2 hour in 5 X SSPE, 50% formamide, 0.1% SDS, 5 X Denhart's solution, and 200 μg/ml denatured herring sperm DNA, then hybridized with 2 x 10^5 dpm/ml of ^{32}P-labeled probe at 42 C for 18 h in the same mixture containing 5% dextran sulfate. The filters were then washed twice with 6 X SSPE, 0.1% SDS at 42 C for 1 h, then once in 1 X SSPE, 0.1% SDS at 42 C for 20 minutes. Equal loading and transfer of RNA was confirmed by quantitation of methylene-blue stained 28S rRNA. Filters were exposed to Fuji-XR film with two Dupont Cronex III instensifying screens at -80 C for 5 days. After autoradiography, the relative intensities of the respective bands were quantitated by laser densitometry (Biomed Instruments, Fullerton, CA) and normalized to the respective densities of the 28S rRNA bands on methylene blue stained filters.

4. Nuclear run-on analysis

The effect of PG on the rate of IGFBP-5 gene transcription was determined by nuclear run-on analysis (Celeno et al., 1989). Briefly, U2 cells were treated with 10 nM PG or with vehicle for 1 and 4 h. The cells were rinsed and scraped into ice-cold phosphate-buffered saline and nuclei were prepared. The nuclei were then resuspended in

transcription buffer (0.6 M KCl, 12.5 mM $MgCl_2$, 2.5 mM of ATP, CTP and GTP) and transcription was allowed to proceed *in vitro* by addition of 100 μCi [^{32}P]-UTP (800 Ci/mmol). Equal amounts of labeled RNA (2×10^6 dpm) were hybridized to 10 μg of alkali-denatured plasmid DNAs which were immobilized on Hybond-N filters. The filters were hybridized in a solution containing 10 mM TES (2-{[tris-(hydroxymethyl)]-methylamino}-ethanesulfonic acid) pH 7.4, 10 mM EDTA, 0.2% SDS, and 0.6 M NaCl at 68 C for 48 h. After hybridization, the filters were washed twice with 6 X SSPE, 0.1% SDS at 42 C for 30 min and once with 1 X SSPE, 0.1% SDS at 42 C for 30 min and autoradiographed with two Dupont Cronex III intensifying screens for three days. The relative intensity of each band on the autoradiographs was quantitated by laser densitometry as described above.

5. Plasmid preparation

A single bacteria colony harboring plasmids or cosmids was grown overnight with shaking at 37 C in an appropriate volume of TB or LB containing 100 μg/ml of amplicilin or 50 μg/ml of kanamycin. The cells were pelleted in a 250 ml polypropylene bottle or 50 ml disposable tube at 10,000 x g for 15 min at 4 C. Plasmid DNA was purified using either Wizard mini-prep or Qiagen maxi kits. All plasmid DNA was stored at 4 C.

a. Wizard mini-preparation

To check for the presence of recombinant plasmid within a given colony and to prepare plasmid for subcloning and DNA sequencing, small scale (3-5 ml) Wizard mini-preparation kits were used as described by the manufacturer's protocol. Briefly, three ml of bacteria culture was pelleted and then resuspended in 200 µl of suspension solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA and 100 µg/ml RNase A). A volume of 200 µl of cell lysis solution (0.2 M NaOH, 1% SDS) was added. The tubes were gently inverted a few times, and then 200 µl of neutralization solution (2.55 M KOAc, pH 4.8) was added and mixed. The mixture was centrifuged in a microcentrifuge at top speed (14,000 x g) for 5 min at room temperature. The supernatant was transferred to a fresh tube and mixed with 1 ml of DNA purification resin by inverting the tube. The mixture was incubated at room temperature for 1 min. The mixture was then loaded onto a mini-column attached to a vacuum manifold. The column was washed twice with 1 ml column wash solution (200 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA). The plasmid DNA was finally eluted with 50 ml of 65 C water.

b. Alkaline lysis preparation

This method was used to prepare cDNA probes and plasmids for nuclear run-on analysis as described (Sambrook et al., 1989). The bacterial pellet was resuspended in 0.5 ml of LB and 5 ml of TENS buffer containing 100 µg/ml RNaseA was added. The mixture was shaken until sticky. After addition of 2.5 ml of 3 M NaOAc pH 5.2, the

mixture was placed on ice. The lysate was centrifuged at 10,000 x g for 20 min at 4 C. The supernatant was transferred to a fresh tube and 10 µl (10 µg/ml) RNase A was added, mixed and incubated at 37 C for 30 min. The mixture was extracted with 10 ml of phenol/chloroform/IAA (24:24:1) by shaking vigorously and placed on ice for 5 min. The mixture was centrifuged at 10,000 x g for 20 min at 4 C. The supernatant was transferred to a new tube and 1/10 volume of 3 M NaOAc pH 5.2 and 15 ml of absolute ethanol were added and mixed. The mixture was then placed at -20 C overnight. Plasmid DNA was pelleted by centrifugation at 10,000 x g for 20 min at 4 C. The DNA pellet was washed twice with -20 C 70% ethanol. The pellet was air-dried for 10-15 min, then resuspended in sterile distilled water.

c. Qaigen maxi-preparation

For preparation of DNA for transfection and for cosmid DNA purification, a Qaigen maxi-preparation kit was used. The purification steps were carried out according to the protocol provided by the manufacturer. Typically, a single colony of bacteria was resupended in 150 ml of TB broth and grown in a 37 C shaking incubator for 12-16 hr. The bacteria suspension was then transferred to a 250 ml centrifuge bottle and centrifuged at 10,000 x g at 4 C for 10 min. The bacterial pellet was resuspended in 10 ml of solution P1 (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 100 µg/ml RNaseA). 10 ml of solution P2 (200 mM NaOH and 1% SDS) was added, mixed by inverting the tube and incubated at room temperature for 5 min. 10 ml of ice-cold solution P3 (3.0 KOAc, pH

5.5) was then added, mixed, and placed on ice for 20 min. The lysate was centrifuged at 30,000 x g for 30 min at 4 C. The Qaigen-500 tip was equilibrated with QBT buffer by gravity flow (750 mM NaCl, 50 mM MOPS, pH 7.0, 15% ethanol, 0.15% Triton X-100). After centrifugation, the supernatant was loaded onto the tip. The tip was washed twice with 30 ml of QC buffer (1.0 NaCl, 50 mM MOPS, pH 7.0, 15% ethanol). The plasmid was then eluted from the tip with 15 ml of QF buffer (1.25 M NaCl, 50 mM Tris-HCl, pH 8.5, and 15% ethanol) and precipitated with 10.5 ml or 0.7 volume of isopropanol. The plasmid DNA was pelleted by centrifugation at 13,000 x g for 30 min at 4 C. The pellet was washed twice with 70% ethanol, air dried for 5 min, and resuspended in sterile distilled water. This preparation typically yielded at least 300 μg of plasmid DNA.

6. Probe isolation, labeling and purification

a. cDNA probe isolation

The human IGFBP-5 cDNA probe was prepared by *Eco*RI digestion of plasmid BP5/5 and electrophoretic separation on 1.0% SeaPlaque, low melting point agarose in 1 x TBE running buffer as described (Sambrook et al., 1989). The gel was stained with ethidium bromide and the cDNA fragments were excised from the gel. The gel containing the cDNA fragment was melted at 70 C for 5 min and an equal volume of DEPC-treated water was added. The gel mixture containing cDNA was stored at -20 C.

b. cDNA probe labeling and purification

cDNA probes were labeled by the random priming method as described (Feinburg et al., 1983) The gel containing the cDNA fragment was melted at 70 C for 5 min. A volume of gel containing 50-100 ng DNA was diluted in DEPC water to a volume of 31 µl, boiled for 5 min and quickly placed on ice for 2 min. The following solutions were added in order to the cDNA solution : 10 µl of 5 x cDNA labeling buffer, 2 µl of DNase-free BSA (10 mg/ml), 5 µl of α-[^{32}P]-CTP (66.7 µCi) and 2 µl of Klenow fragment (5 U/µl). The mixture was incubated at 37 C for 2-12 h.

Labeled DNA was purified using NACS chromatography as described by the manufacturer. The column was hydrated with 3 ml of 2 M NaCl/TE and equilibrated with 5 ml of 0.5 M NaCl/TE. The probe was then loaded onto the column and washed twice with 5 ml of 0.5 M NaCl/TE. The labeled probe was finally eluted with 400 µl of 0.2 M NaCl/TE. Labeling efficiency was measured by liquid scintillation counting.

c. Oligodeoxynucleotide probe labeling and purification

Single stranded or double stranded oligodeoxynucleotide probes were end-labeled with T4 kinase as described (Ausubel et al., 1994). The 10 µl of labeling reaction contained 50 -100 ng of oligodeoxynucleotide, 1 µl of 10 x TMDSE, 200 µCi γ-[^{32}P]-ATP and 1 U T4 polynucleotide kinase. The mixture was incubated at 37 C for 1 h and labeled oligodeoxynucleotide was purified using a MERmaid kit as suggested by the manufacturer. To 10 µl of labeling reaction, 30 µl of High Salt Binding Solution and

5 µl of Glassfog were added, vortexed, and incubated at room temperature for 5-15 min. The Glassfog was then pelleted by brief centrifugation. The pellet was resuspended and washed twice with 300 µl of ethanol wash solution and air-dried for 5 min. The labeled oligodeoxynucleotide was eluted twice with 50 µl of DEPC water. The labeling efficiency was determined by liquid scintillation counting.

7. Isolation of IGFBP-5 genomic clones

The human placental genomic cosmid library (pWE15) was resuspended in 5 ml of TB and spread on three 231 cm^2 BA-S supported nitrocellulose filters. The filters were then placed on TB/Amp (TB agar containing 100 µg/ml amplicilin) plates (master plates). Plates were incubated at 37 C until colonies were 0.2 mm in diameter. Nitrocellulose or Whatman 541 replica filters were made from each master nitrocellulose filter. The replica filters were allowed to grow on TB/Amp plates at 37 C for 6-8 h. Colonies on replica filters were lysed and fixed by sequential soaking in: 0.5 N NaOH, and 1 M Tris-HCl (pH 8.0)/ 1.5 M NaCl. The filters were soaked for 5 min in each solution and finally rinsed in 2x SSC to remove cell debris. Filters were then cross-linked at 1,200 µJ/mm^2 (optimal crosslinking setting), fixed in 95 % ethanol,and baked at 80 C for 30 min in a vacuum oven.

A mixture of two oligodeoxynucleotides corresponding to regions of the 3'-untranslated region of human IGFBP-5, 5'-CCCAGGTAAGAGGAGAGGAA-3' and 5'-CTGGCTAGAGGAGGAGACA-3', were used to screen the library. These were

labeled with γ-[^{32}P] ATP using T4 Polynucleotide Kinase. Whatman filters No. 541 were prehybridized in 5 X Denhart's, 0.01 M EDTA and 0.5 % SDS at 42 C for 2 h. The filters were hybridized in 5 X SSPE, 5 x Denhart's, 0.1 % SDS, 250 μg/ml of yeast tRNA, and 2-5 x 10^6 dpm of probe per ml of hybridization solution at 42 C overnight. Filters were washed with 6 x SSPE/ 0.1 % SDS at 42 C for 15 min, 6 x SSPE/ 0.1 % SDS at 42 C for 60 min, and 6 x SSPE/ 0.1 % SDS at 55 C for 30 min. Filters were sealed and exposed to Fuji-XR film at -80 °C for 24 h. 4-6 x 10^6 recombinant clones were screened at high density.. All positive clones were later plated at low density and rescreened with ^{32}P-labeled human IGFBP-5 cDNA. The purified cosmid clones which hybridized strongly to human IGFBP-5 cDNA were picked and rescreened. A single positive clone was grown up in liquid culture. DNA was isolated using a Qiagen MaxiPrep kit (Qaigen, Chatworth, CA). The cosmid DNA was digested with *Eco*RI, *Pst*I, *Sac*I, *Bam*HI and *Hind*III, and subjected to Southern Analysis using a ^{32}P-labeled oligodeoxynucleotide, 5'-ACTCTCGCTCTCCTGCCCCA-3', corresponding to the 5'-untranslated Exon I region of human IGFBP-5. The cosmid fragments hybridizing to this oligodeoxynucleotide were gel purified, subcloned into pGEM3Zf(-) (Promega, Madison, WI) and sequenced by the dideoxy-chain termination method using a commercial kit, Sequenase 2.0 (USB) as described by the manufacturer.

8. Southern Blot Analysis

Southern analysis of cosmid DNA was carried out on a 1% agarose gel. The DNA was denatured by immersing the gel in 1.0 M NaCl/0.5 M NaOH for 20 min, twice. The gel was neutralized by soaking in 0.5 M Tris-HCl (pH7.5) /1.5 M NaCl for 20 min, twice. DNA was transferred to MagnaGraph membranes (MSI, Nestboro, MA) using 10 X SSC as the transfer buffer. The membranes were UV cross-linked, prehybridized (6 X SSPE, 10 X Denhart's solution, 0.5% SDS, 100 μg/ml denatured herring sperm DNA) at 42 C for 2 h, hybridized (6 X SSPE, 10X Denhart's solution, 0.5% SDS, 50% formamide, 200 μg/ml denatured herring sperm DNA) at 42 C for 18 h, washed and exposed to X-ray film. For cDNA and genomic fragment probes, filters were washed with 1 X SSC, 0.1% SDS at 42 C for 1 h, followed by 0.1 X SSC, 0.1 % SDS at 55 C for 1 h. For oligodeoxynucleotide probes, filters were washed with 6 X SSC, 0.1% SDS at 42 C for 1 h, followed by 6 X SSC, 0.1% SDS at 55 C for 1 h.

9. Primer extension analysis

Total RNA from U2 cells grown in DMEM + 10% CS was isolated and primer extension analysis was carried out as described. Briefly, 50 μg of total RNA was mixed with 5 x 10^4 cpm of a ^{32}P-labeled oligodeoxynucleotide located 65 bp from the putative transcriptional start site, (5'-GTGAAAACGGAGGAGGGGTAATGAAAAG GC-3'). The RNA mixture was then denatured at 80 C for 5 minutes and allowed to anneal at 50 C for 18 h. The primer was then extended using 200 Units of Superscript RNase H$^-$

reverse transcriptase (GIBCO/BRL, Gaitherburg, MD) at 42 C for 90 minutes. After ethanol precipitation, the extended products were analyzed on an 8% urea-polyacrylamide sequencing gel and the size was determined by comparing the band migration rate to that of a dideoxysequencing standard on the same gel.

10. Subcloning

A 4.6 Kb *Eco*RI fragment which hybridized very strongly to both the IGFBP-5 cDNA and the 5'-UTR region oligodeoxynucleotide was subcloned into pGEM-3Zf(-). To prepare the vector, 10 µg of vector was digested at 37 C for 2 hr with *Eco*RI as suggested by the manufacturer, and dephosphorylated for 1 hr with calf intestinal alkaline phosphatase at 37 C. The linearized plasmid was purified using a Wizard PCR column. To prepare the insert, 50 µg of IGFBP-5 cosmid DNA was digested for 2 hr with *Eco*RI, and separated on a 1 % low melting point agarose gel. The 4.6 kb fragment was excised and melted by heating to 68 C for 5 min. 25 µl of agarose containing the DNA fragment, 18 µl of 10 mM Tris-HCl (pH 7.5), 5 µl of 10 X T4 DNA ligase buffer, 1 µl of purified vector and 1 µl of T4 DNA ligase were mixed gently by flicking the tube. The ligation reaction was incubated at room temperature for at least 2 hr. At the end of the incubation period, the reaction was remelted by heating to 68 C for 5 min and placed in a 37 C waterbath. An aliquot of 10 µl of the ligation reation was mixed with 10 µl of 10 mM Tris-HCl (pH 7.5) prewarmed to 40 C and placed in the 37 C waterbath. For transformation, 2 µl of diluted ligation mixture and 2 µl of 0.5 M ß-mercaptoethanol were

mixed with 50 µl of INVαF ' competent bacteria in a 2 ml centrifuge tube. The mixture was incubated on ice for 30 min, and heated to 42 C for 1 min. Immediately after heating, 450 µl of SOC medium was then added to the mixture. It was placed in a 37 C shaking incubator for 1 hr. Aliquots of 50 and 200 µl of the transformed competent cell mixture were plated on 10 ml TB plates containing 100 µg/ml of amplicilin. X-Gal was dissolved in dimethylformamide and a small volume (25-30 µl) was added to the plate to the final concentration of 50 µg/ml.

White positive clones lacking ß-galactosidase activity were picked, plasmid DNA was isolated, and DNA was digested with *Eco*RI to confirm the insert size. One positive clone was then picked, grown in 150 ml of TB/amp broth, and plasmid DNA was isolated using the Qaigen Maxi-Prep kit. This plasmid (clone 3E3) was used for DNA sequencing and was further subcloned for promoter analysis.

11. Polymerase chain reaction (PCR)

PCR (Innis et al., 1990) was used to facilitate the subcloning of specific regions of the IGFBP-5 promoter. The reaction was performed in a 50 µl reaction volume containing 1 X NEB Vent buffer, 20 ng of plasmid DNA containing the IGFBP-5 promoter, 20 pmol each of IGFBP-5 promoter sense and antisense oligodeoxynucleotides, and 250 µM of each dNTP. The reaction was mixed and overlaid with 50 µl of light mineral oil (USB, Cleveland, OH). The PCR reaction was performed on a Coy thermocycler (Coy, Ann Arbor, MI). The reaction was heated to 100 C for

5 min and cooled to the annealing temperature at 58 C for 2 min. Then, 2 units of Vent (exo$^-$) was quickly added to the reaction and mixed by flicking the tube. The tube was placed back into the thermocycler and the reaction was then allowed to proceed for 32 cycles using an extension temperature of 74 C for 30 sec, a denaturation temperature of 98 C for 1 min and an annealing temperature of 58 C for 1 min. At the end of the 32nd cycle, the reaction was further incubated at 74 C for 7 min . Subsequently, 50 µl of TE saturated chloroform/IAA (24:1) was used to extract the mineral oil. To access production of the desired product, 20 µl of the aqueous phase was subjected to electrophoresis. DNA (typically, about 1 µl from 50 µl PCR reaction or 20-50 ng of DNA) in the reaction containing the desired DNA fragment was then directly subcloned without further purification into the pCRII plasmid using a TA cloning kit (Invitrogen, San Diego, CA) as described by the manufacture. Typically, 2 µl of PCR product, 1 µl (20 ng) of pCRII vector, 1 µl of 10 X ligation buffer, and 6 µl of distilled steriled water was added to a 1.5 ml tube. The ligation mixture was incubated at 12 C, overnight. One µl of ligation mixture was then used to transform INVα F' competent bacterial cells.

12. Reporter plasmid construction

Seven IGFBP-5 promoter-reporter constructs containing 753, 461, 345, 325, 252, 162, and 124 bp of IGFBP-5 of the 5'-flanking region (Figure 3) were prepared using selected restriction enzyme sites or by using PCR with specific primers to selectively produce the desired promoter region. The fragments were subcloned into a

chloramphenicol acetyltransferase (CAT) reporter gene vector pJFCAT1 (Figure 4). Detailed strategies for subcloning of each construct are described below. The name of each subclone is shown in parentheses.

a. pCAT753 was constructed by cutting the 3E3 plasmid (See subcloning section) with *Pst* I. The 776 *Pst* I fragment which contained 753 bp of IGFBP-5 gene 5'-flanking region and 23 bp of IGFBP-5 gene's 5'-UTR was gel purified and subcloned into the *Pst* I site of pJFCAT1.

b. pCAT461 was constructed by cutting pCAT753 with *Hind*III to release an insert of 490 bp containing 461 bp of the IGFBP-5 gene's 5'-flanking region. This *Hind*III fragment was then subcloned into the *Hind*III site of pGEM-7Zf(-) (pGH). A clone which contained an antisense insert (pGH7) was picked. Subsequently, pGH7 was digested with *Bam*HI and *Pst* I and the fragment was subcloned in the sense orientation into the *Bam*HI and *Pst* I sites of pJFCAT1.

c. pCAT345 was constructed by using specific primers of 20 bp in length to amplify the region from +345 to -23 of the hIGFBP-5 gene. The 368 bp PCR product was then subcloned into the pCRII vector (pCR345) using TA-cloning (Invitrogen, San Diego, CA). The insert was flanked with *Eco*RI sites of the pCRII vector, and was excised with *Eco*RI, gel purified and subcloned into the *Eco*RI site of pGEM7Zf(-) (pG345). An insert in the sense orientation was then subcloned from pG345 into the *Sph*I and *Pst* I sites of pJFCAT1. The insert carried *Xba* I, *Xho* I, and *Eco*RI sites at the 5'-end.

Figure 3. Schematic representation of hIGFBP-5 deletion promoter reporter constructs. A partial restriction map of the human IGFBP-5 gene *Eco*RI fragment containing the 5' flanking region is shown at the top. Thin vertical lines represent putative PRE half-sites. The solid box represents CACCC sequences, the thick vertical line represents the TATA box, and the open and striped boxes represent the 5' untranslated region and protein coding region of Exon 1. The *Pst*I site within Exon 1 was used to ligate the 5' flanking region fragment into the CAT reporter gene pJFCAT1. Constructs shown below the restriction map were composed of 753, 461, 345, 325, 252, 162, and 124 bp of IGFBP-5 5'-flanking region and contained 5, 4, 2, 2, and 0 putative PRE half-sites.

EcoRI Promoter Fragment

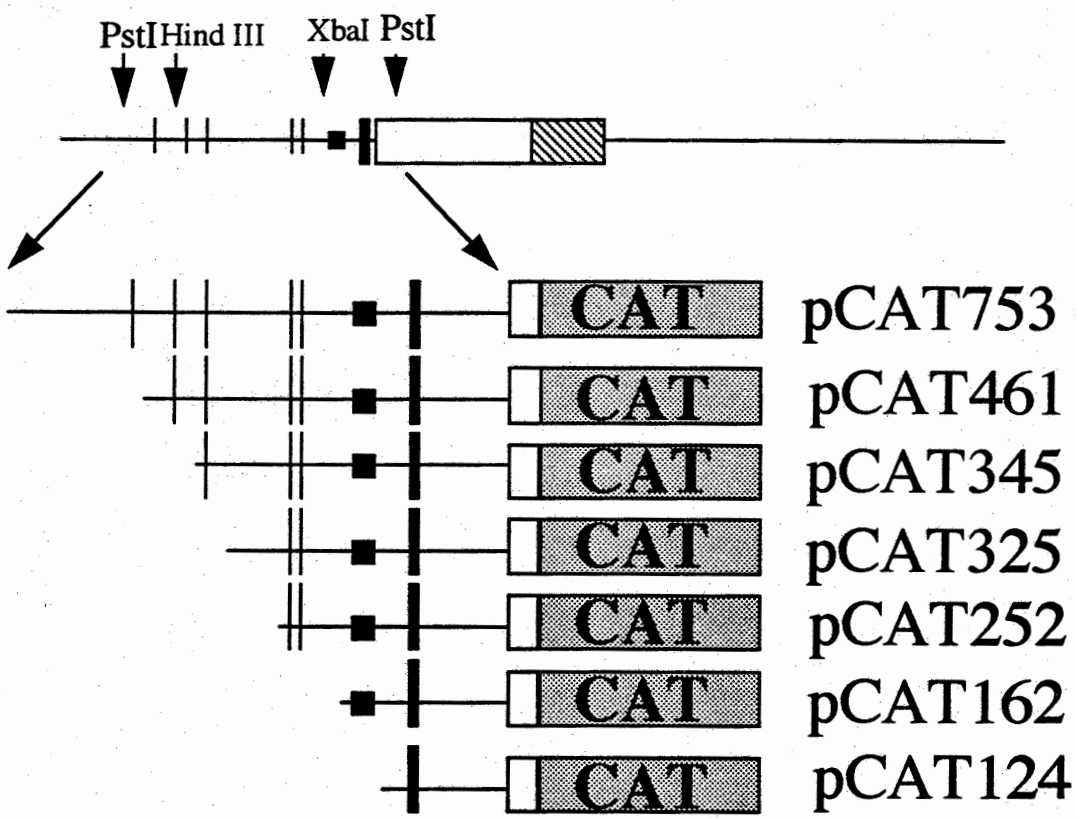
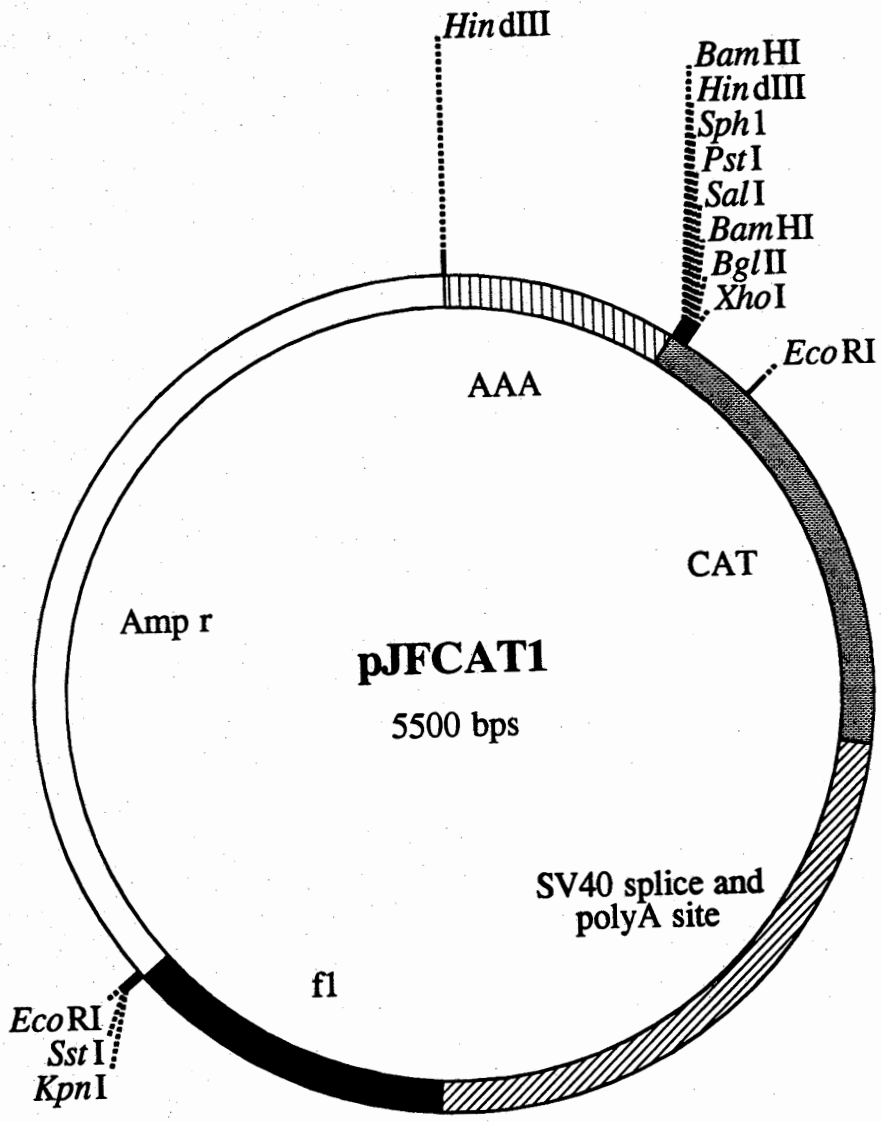


Figure 4. Map of promoterless vector, pJFCAT1. AAA represents the SV40 polyadenylation site trimer cassette. CAT represents the chloramphenicol acetyltransferase gene. f1 represents the phage f1 origin of replication. Amp r represents the ß-lactamase gene conferring amplicilin resistance. For the construction of hIGFBP-5 promoter reporter constructs, DNA fragments containing hIGFBP-5 gene 5'-flanking regions were subcloned into the multiple cloning sites of the vector.


HindIII
BamHI
HindIII
Sph1
PstI
SalI
BamHI
BglII
XhoI
EcoRI
AAA
CAT
Amp r
pJFCAT1
5500 bps
SV40 splice and
polyA site
f1
EcoRI
SstI
KpnI

d. pCAT325 was constructed the same way as pCAT345 except the PCR product was the fragment +325 to -23 of the hIGFBP-5 gene.

e. pCAT252 was constructed the same way as pCAT345 except the PCR product was the fragment +252 to -23 of the hIGFBP-5 gene.

f. pCAT162 was constructed by cutting pCAT461 with *Xho*I and *Xba*I. The fragment was subcloned into the *Xho*I and *Xba*I site of pGEM7Zf(-) (pG162). The insert from pG162 was released by cutting with *Sph*I and *Pst*I and subcloned into the *Sph*I and *Pst*I sites of pJFCAT1.

g. pCAT124 was constructed the same way as pCAT345 except the PCR product was the fragment +124 to -23 of hIGFBP-5 gene.

To investigate the functionality of the TATA box at position 1052, pCAT643 was made. pCAT643 was constructed by cutting 3E3 plasmid with *Pst*I The 643 bp *Pst*I fragment which contained positions -1390 to -747 of the IGFBP-5 5'-flanking region was gel purified and subcloned into *Pst*I site of pJFCAT1.

For pCAT162mut, a synthetic 44-mer double stranded oligodeoxynucleotide with overhanging *Sph*I ends was made. The sense strand sequence is : 5'-CTCTAGAAGGC CTCT**C**<u>**AAA**</u>**ACC**<u>**AAA**</u>**ACCCC**GTGTGAGTTGCATG-3' (altered nucleotides underlined). The oligodeoxynucleotides were annealed by boiling in a 200 ml water bath and cooling to 22 C over 2 h. The double stranded oligodeoxynucleotide was inserted in the sense orientation into the *Sph*I site of pCAT124 upstream of the IGFBP-5 5'-flanking region. The construction of pCAT162mDi and pCAT162mPr were the same as

pCAT162mut except a different set of oligdeoxynucleotides which had CACCC box mutations at different positions were used. In pCAT162mDi, the sequence CCCACCCCCACCCC was mutated to AAAACCCCCACCCC. In pCAT162mPr, the sequence was mutated to CCCACCAAAACCCC.

The sequence and orientation of all promoter constructs used were confirmed by sequence analysis using a commercial kit, Sequenase 2.0 (USB, Cleveland,OH).

13. DNA transfection

To determine basal promoter activities of the IGFBP-5 promoter constructs, 3 x 10^5 U2 cells were seeded in 60 mm dishes in DMEM supplemented with 10% CS. After 18 h of incubation, cells were rinsed once with DMEM and 1.6 ml of DMEM containing 12 µl of LipofectAMINE (GIBCO/BRL, Gaithersburg, MD) and 6 µg of Qiagen purified DNA (5 µg of CAT construct + 1 µg of pCMVß-gal) was added to the cells. The cells were incubated for 4 hr at 37 C, in 95% air-5% CO_2. Medium was then replaced with DMEM supplemented with 10% CS and cells were incubated for 48 hr before ß-galactosidase and CAT activities were assayed. To evaluate the effect of PG on promoter activity, U2 cells were grown in phenol-red free DMEM supplemented with 5% CD-FBS for 72 h before transfection. Cells were then plated at 2 x 10^5 cells per 60 mm dish, and transfected as above. When progesterone receptors were overexpressed, U2 cells were transfected with 2 µg of phPR-A or phPR-B plasmid , 3 µg of the IGFBP-5 promoter construct and 1 µg of pCMV-ßgal. After transfection, media was then replaced

with phenol-red-free DMEM supplemented with 2% CD-FBS for 24 h. Medium was replaced again with phenol-red-free DMEM supplemented with 2% CD-FBS, plus 10 nM PG or solvent, for another 24 h. Cells were lyzed with reporter lysis buffer (Promega, Madison, WI) as recommended by the manufacturer (see section below). CAT and ß-galactosidase activities were determined as described below and CAT activity was expressed per unit of ß-galactosidase activity. Statistical analysis of differences between groups was determined by ANOVA using Systat software (Systat Inc., Evanston, IL).

14. Cell extract preparation

At the end of the incubation period, the medium was removed and cells were washed twice with PBS. 500 µl of 1X Reporter lysis buffer (Promega) was added to cover the cells in 60 mm dishes. The cells were incubated at room temperature for 15 min, scraped and transferred to a microfuge tube and placed on ice. Cell debris was pelleted for 2 min at 4 C by centrifuging at top speed in a microfuge. The supernatant was transferred to a fresh microfuge tube and assayed for ß-galcatosidase and CAT activities immediately or stored at -80 C for assaying at a later time.

15. ß-Galactosidase assay

The ß-galactosidase assay was performed as described by Rosenthal (Rosenthal, 1987). The standard assay was performed in a microtiter plate by adding 50 µl of a 2 X assay buffer which contains the substrate ONPG (o-nitrophenyl-ß-D-galactopyranoside)

to 50 µl of the sample. Samples were incubated at 37 C for 30 min. The reaction was terminated by adding 150 µl of 1 M sodium carbonate and A_{420} was determined with a plate reader (SLT Lab Instruments, Austria). A standard curve was developed by plotting A_{420} versus concentrations of ß-galactosidase standard (GIBCO-BRL, Gaithergburg, MD). The ß-galactosidase activities of cell extract samples were determined from the standard curve.

16. CAT assay

Cell extract samples were heated to 60 C for 10 min. 70.5 µl of 0.25 M Tris-HCl (pH7.6), 0.5 µl of [^{14}C]-chloramphenicol (0.025 µCi/ml), 5 µl of 5 µg/ml n-butyryl Co A, and 50 µl of cell extract were mixed and incubated at 37 C for 2 hr to overnight. Subsequently, 300 µl of xylene was added to the mixture and vortexed for 30 sec. The mixture was centrifuged at maximum speed in a microcentrifuge at room temperature for 3 min. The upper phase was then transferred to a new microfuge tube containing 100 µl of 0.25 M Tris-HCl (pH7.6). The mixture was then back-extracted by vortexing and centrifuging as described above. The back extraction step was repeated twice, then, 200 µl of upper phase was transferred to a scintillation vial containing 2 ml of scintillation cocktail (ScintiSafe EconoL, Fisher Chemical, Fairlawn, NJ). The radioactivity was measured in a scintillation counter. CAT activity in each sample was reported as CPM per unit of ß-galactosidase activity.

17. Nuclear extract preparation

U2 cell nuclear extracts were prepared as described by Dignam et al (Dignam et al., 1983). U2 cells were plated in thirty 100 mm dishes at 4 x 10^5 cells in phenol-red free DMEM containing 5% CD-FBS and incubated at 37 C in a humidified incubator with 95% air and 5% CO_2 overnight. The medium was then removed and replaced with phenol-red free DMEM containing 2% CD-FBS and incubated for 24 hr. When cells reached 80% confluence, thirty of 100 mm dishes were extracted. Before harvesting the cells, medium was removed and cells were rinsed once with 1-2 ml of cold 1 X PBS. One ml of ice cold 1 X PBS was then added per dish. Cells were harvested by scraping with a rubber policeman , collected in a 50 ml polypropylene tube, and centrifuged at 1,800 rpm (700 x g) at 4 C for 10 min in an IEC Centra-7R refrigerated centrifuge (Fisher Sciencetific, Pittsburgh, PA). The cell pellet was washed once in cold 1 X PBS and resupended in 5 ml of ice cold Detergent lysis buffer (cells should be resuspended at 1 X 10^6 to 3 X 10^6 cells per ml of buffer). The cell suspensions were then spun at 2,000 rpm (900 x g) at 4 C for 10 min in an IEC Centra-7R refrigerated centrifuge. The supernatant was removed and cells were resuspended in 10 ml of cold RSB (see Section C). The cell suspensions were then spun at 1500 rpm (500 x g) at 4 C for 4 min in an IEC Centra-7R refrigerated centrifuge. The supernatant was removed and the pellet was resuspended in a volume of extraction buffer A equivalent to the volume of the nuclear pellet by mixing with a 1000 µl pipette tip cut off at the end to make a larger opening. The mixture was then transferred to an microfuge tube and a volume of

extraction buffer B equivalent to the volume of nuclear pellet was added. Typically, 0.5 to 1.0 ml of total extract was subjected to further treatment. The tube was rocked in the cold room for 30 min. The nuclear lysate was then transferred to six 5 X 20 mm polyallomer centrifuge tubes containing 150 µl in each tube and centrifuged in an Airfuge Ultracentrifuge (Beckman Intrument, Palo Alto, CA) in a cold fix-angle aluminum rotor at maximum speed (17,000 x g) for 15 min at 4 C. The supernatant was transferred to a Slide-A-Lyzer 10K dialysis cassette (Pierce, Rockford, IL) and dialyzed for 2 hr. in 100 ml of cold buffer C in the cold room with one buffer change at the end of the first hour. The dialysate was transferred to a microfuge tube and centrifuged at 14,000 rpm in a microfuge in the cold room for 10 min to pellet cell debris. The supernatant was then transferred to a microfuge tube, aliquoted into small volumes (50 µl per aliquot), fast frozen in liquid nitrogen, and stored at -70 C.

The nuclear extract protein concentration was determined by Bradford protein assay (Biorad, Richmand, VA) with BSA as a standard as described in the product's manual. The standard assay was performed in a microtiter plate by adding 100 µl of 40% Bradford dye solution to the nuclear extract diluted in 100 µl of buffer C or to BSA standards (0.1 to 5 µg in 100 µl of buffer C). Samples were incubated at room temperature for 5 to 10 min. The A_{550} was determined with a plate reader (SLT, Lab Instruments, Austria). A standard curve was developed by plotting A_{550} versus concentrations of protein standard. The nuclear extract protein concentration was determined from the standard curve.

18. Electrophoretic mobility shift assay (EMSA)

For the EMSA, the binding reaction contained 1X binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, and 10 mM Tris-HCl, pH 7.5), 0.05 mg/ml poly (dI-dC), 1.5 µl (3 µg protein) of nuclear extract, and 1 µl (50,000 cpm) of ^{32}P-labeled double stranded oligodeoxynucleotides in a total volume of 20 µl (typically, 4 µl of 5 X binding buffer, 1 µl of 1mg/ml poly (dI-dC), 1.5 µl of nuclear extract, 1 µl of labeled double stranded oligodeoxynucleotides and water to the total volume of 20 µl). For cold competition experiments, nuclear extract was preincubated with unlabeled double stranded oligodeoxynucleotides for 10 min at room temperature before the addition of the labeled double stranded oligodeoxynucleotides.

Purified PR-A and PR-B were obtained from a baculovirus expression system and purified as described (Onate et al., 1994). To evaluate PR interactions with U2 cell nuclear factors, different concentrations of PR proteins were preincubated with 5 µg of U2 cell nuclear extract protein before the addition of labeled double stranded oligodeoxynucleotides. The reaction mixture was incubated at room temperature for 20 min and analyzed by electrophoresis using a 5% polyacrylamide-0.125% bis-acrylamide gel in 0.25 x TBE pH 8.0. For supershift experiments, 1 µg of anti-PR antibody AB52 (Onate et al., 1994) was added to the reaction and incubated on ice for an additional 15 min before loading onto the gel. After electrophoresis, the gel was dried and autoradiographed using Kodak X-OMAT or Biomax MS film and Dupont Cronex III intensifying sceens.

C. Solutions

1. Common molecular biology

a. cDNA labeling mix: 1M HEPES, pH 6.6, 0.3 M 2-mercaptoethanol, 15 units hexanucleotides, 0.2 mM each dNTP(dATP, dGTP, dGTP, dTTP), 0.025 M Tris-HCl, pH 8.0, 2.5 mM $MgCl_2$.

b. 10 X TMDSE: 0.01 M Tris-HCl, pH 7.6, 0.1 M $MgCl_2$, 0.05 M DTT, 1 mM spermidine and 1 mM EDTA.

c. Deionized formamide: Formamide was melted at room temperature and deionized with 1 g AG 501 X 8 resin (BioRad) per 10 ml of formamide by stiring for 30 min at room temperature. The formamide-resin suspension was filtered twice with Whatman #1 filter paper, aliquoted into 50 ml polypropylene tubes and stored at -20 C.

d. 50 X Denhart's solution: 1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin. The solution was filtered through a disposable 0.22 micron Nalgene filter.

e. DEPC water: 0.1% diethlyl-pyrocarbonate (DEPC) was added to double-distilled water to 0.1% v/v, shaken, and incubated for at least 1 hr. The DEPC treated water was then autoclaved.

f. Luria broth (LB): 0.1% Bacto-tryptone, 0.05 % bacto-yeast extract, 0.1% NaCl, pH 7.1. This was autoclaved, then cooled to room temperature before use.

g. Terrific broth (TB): 1.2% Bacto-tryptone, 2.4% yeast extract and 0.4% glycerol, potassium phosphate, pH 7.4. This was autoclaved, then cooled to room temperature before use.

h. Methylene blue staining solution: 0.02% methylene blue and 0.3 M sodium acetate, pH 5.5. The solution was stored in an amber bottle to protect from light.

i. 10 X MOPS: 0.2 M 3-(N-morpholino) propanesulfonic acid, 5 mM sodium acetate, 5 mM EDTA, pH 7.0. This was autoclaved, then cooled to room temperature before use.

j. Northern sample loading buffer: 720 µl deionized formamide, 320 µl 10 X MOPS, 260 µl 37% stock deionized formamide solution, 100 µl glycerol, 4 mg bromphenol blue, 4 mg xylene cyanol and 100 µl DEPC water.

k. Pre-GIT extraction solution: 4 M guanidium thiocyanate, 0.025 M sodium citrate and 0.5 % N-lauryl sarcosine.

l. GIT extraction solution: 360 µl of 2-mercaptoethanol per 50 ml pre-GIT extraction solution, added within 24 hr of use.

m. 10 X NEB Vent Buffer: 0.1 M KCl, 0.2 M Tris-HCl, pH 8.8, 0.1 M $(NH_4)_2SO_4$, 0.02 M $MgSO_4$, 1% Triton X-100.

n. 20 X SSPE: 3.6 M NaCl, 0.03 M $NaHPO_4$, 0.02 M EDTA, pH 7.4, autoclaved.

o. 20 X SSC: 3.0 M NaCl and 3.0 M sodium citrate, pH 7.0, autoclaved.

p. 50 X TAE: 2 M Tris-acetate pH 8.0 and 0.05 EDTA, autoclaved.

q. 10 X TBE: 0.4 M Tris-HCl, pH 8.0, 0.4 M boric acid and 0.012 M EDTA, pH 8.3, autoclaved.

r. TE buffer: 0.01 M Tris-HCl, pH 8.0, 1 mM EDTA, autoclaved.

s. TENS: 0.1 M NaOH, 0.01 M Tris-HCl, pH 8.0, 1 mM EDTA and 0.1 % SDS.

t. ß-Galactosidase assay 2 X buffer: 120 mM Na_2HPO_4, 80 mM NaH_2HPO_4, 2 mM $MgCl_2$, 100 mM ß-mercaptoethanol, 1.33 mg/ml ONPG.

2. Solutions for nuclear extract preparation

a. 1 X PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4, sterile filtered.

b. TKM buffer: 50 mM Tris-HCl pH 7.5, 50 mM KCl, and 15 mM $MgCl_2$, autoclaved.

c. Aprotinin stock: 2 mg/ml in water, aliquoted in 1 ml volume and stored at -20 C.

d. Leupeptin stock: 4 mg/ml in water, aliquoted in 1 ml volume and stored at -20 C.

e. Pepstatin stock: 2.5 mg/ml in methanol, aliquoted in 1 ml volume and stored at -20 C.

f. PMSF stock: 15 mg/ml (100 µM) in isopropanol aliquoted in 1 ml volume and stored at 4 C.

g. Detergent lysis buffer: 50% TKM, 30% sucrose, and 0.5% NP-40. For each 5 ml of the buffer, 5 µl each of pepstatin stock, aprotinin stock, and leupetin stock, and 25 µl of PMSF stock is added immediately before use.

h. RSB buffer: 0.1 M Tris, pH 7.4, 10 mM NaCl, and 3 mM $MgCl_2$. For each 5 ml of the buffer, 5 µl each of pepstatin stock, aprotinin stock, and leupetin stock, and 25 µl of PMSF stock, is added immediately before use.

i. Buffer A: 20 mM HEPES, pH 7.9, 2 mM $MgCl_2$, 200 mM NaCl, 0.2 mM EGTA, 0.2 mM EDTA, 0.5 mM DTT, 5% glycerol, 0.5 mM PMSF, 2 µg/ml aprotinin, 5 µg/ml leupeptin, 5 µg/ml pepstatin.

j. Buffer B: Same as buffer A except that it includes 600 mM NaCl.

k. Buffer C: Same as buffer A except that it includes 100 mM NaCl, and 20% glycerol.

III. RESULTS

A. Effects of PG on hIGFBP-5 mRNA Levels

U2 cells were selected for this study because both IGFBP-5 expression and transfection efficiency were higher in this cell type than in other human osteoblast-like cell lines. 10 nM PG treatment for 6 h increased IGFBP-5 mRNA levels to 240% of control (Figure 5). Stimulatory effects of PG on steady state IGFBP-5 mRNA levels were difficult to demonstrate after longer treatment times since IGFBP-5 mRNA levels increased with time in control cultures such that the relative effect of PG after 24 h of treatment was less than 150% of control (Figure 5). In time course studies, 10 nM PG treatment for as little as 2 h increased IGFBP-5 mRNA to 150-200% of control. This stimulatory effect of PG on IGFBP-5 mRNA levels was not unique to U2 cells or related to the transformed phenotype. In previous work (Lempert et al., 1992) showed that treatment of MG63 human osteosarcoma cells and normal human osteoblast-like cells with 10 nM PG for 2 h increased IGFBP-5 mRNA levels to 270% and 500% of control, respectively.

To determine if the effect of PG to increase IGFBP-5 mRNA accumulation required new protein synthesis, U2 cells were treated concurrently with 10 nM PG and 5 μg/ml cycloheximide. Cycloheximide did not alter the PG stimulated increase in IGFBP-5 mRNA levels, suggesting that PG did not induce production of other proteins which were required for subsequent induction of IGFBP-5 expression (Figure 6). These data support the hypothesis that PG directly induces IGFBP-5 gene transcription.

Figure 5. Time course of increased IGFBP-5 mRNA levels after PG treatment. U2 cells were treated with 10 nM PG or vehicle control for the time intervals indicated. Total RNA was extracted and subjected to Northern analysis. The amount of 25 μg of total RNA was run in each lane. The relative densities of IGFBP-5 mRNA transcript bands on the autoradiographs are indicated as % control measured by laser densitometry.

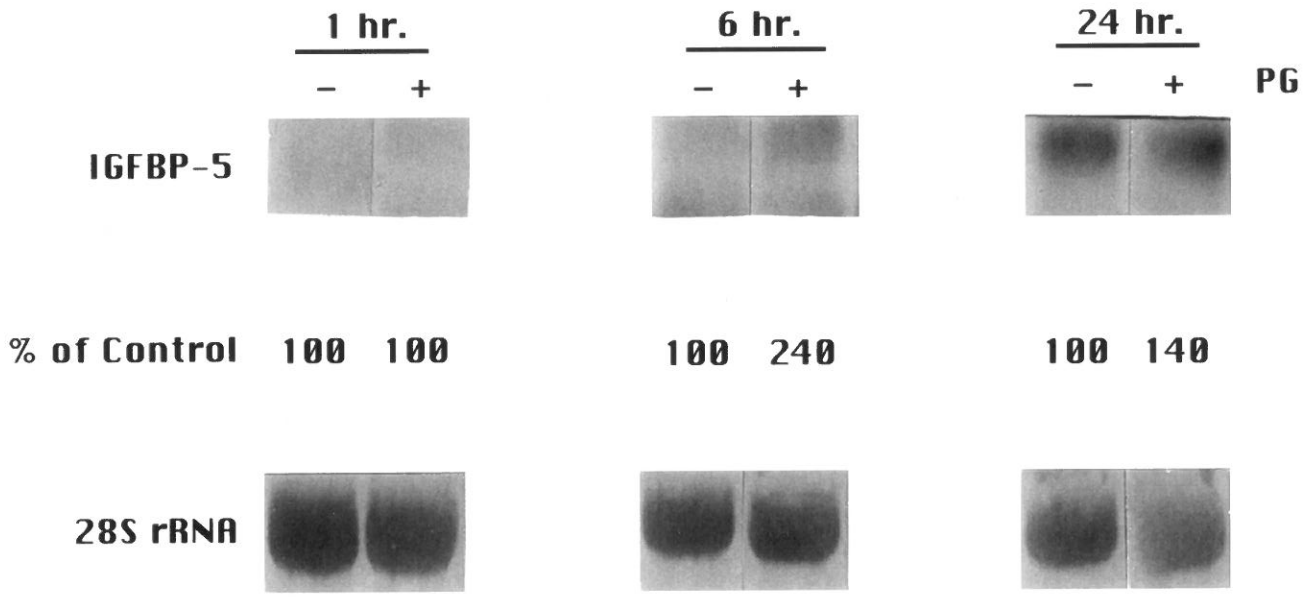

1 hr.
6 hr.
24 hr.
– + – + – +
PG
IGFBP-5
% of Control 100 100 100 240 100 140
28S rRNA

Figure 6. Effect of cycloheximide on the PG stimulated increase in IGFBP-5 steady state mRNA levels. U2 cells were treated concurrently with 10 nM PG and 5 μg/ml cycloheximide, a protein synthesis inhibitor, for 4 h prior to RNA extraction. The relative densities of IGFBP-5 mRNA transcript bands on the autoradiographs are indicated as % control measured by laser densitometry. Cycloheximide, at concentrations which inhibit 90% of new protein synthesis, minimally altered the PG stimulated increase in IGFBP-5 mRNA levels.

IGFBP-5
Control
PG
PG + CHX
% of Control
100
280
240
28S rRNA

B. Effect of PG on the Rate of hIGFBP-5 Gene Transcription

The effect of PG to increase IGFBP-5 mRNA levels within 2 h suggested that PG increased the rate IGFBP-5 gene transcription. To further investigate the mechanism for PG action, the effect of PG on the rate of IGFBP-5 gene transcription was determined by nuclear run-on analysis. Nuclear RNA transcripts (hnRNA) were extracted after treatment with 10 nM PG for a period of time and transcription was allowed to proceed in isolated nuclei in the presence of [^{32}P]-UTP. The labeled RNA was then hybridized to cDNA plasmids of genes of interest which were immobilized on a nylon membrane. Genes which were transcribed at higher rates bound more labeled hnRNA to the immobilized cDNA plasmids and demonstrated stronger band intensities. The rates of gene transcription were then estimated by comparison of band intensities of the genes of interest to those of a gene which was constitutively expressed (ß-actin). PG treatment for 1 h increased IGFBP-5 gene transcription to 400% of control while the rate of ß-actin gene transcription was not significantly affected (Figure 7). After 4 h of PG treatment, the IGFBP-5 transcription rates in both the treated and control groups were less compared to that found after 1 h of PG treatment.

C. Isolation of the hIGFBP-5 Gene

To begin to determine the mechanism by which PG increased the rate of IGFBP-5 gene transcription, the human IGFBP-5 (hIGFBP-5) gene was cloned and the proximal region sequenced. From 4-6 x 10^5 gemomic cosmid clones, one clone was isolated which

hybridized intensely to both the 3'-untranslated region oligodeoxynucleotide mixture (Figure 8). This cosmid contained an insert of approximately 50 kb. The insert was characterized by Southern analysis using hIGFBP-5 cDNA or an oligodeoxynucleotides corresponding to +28 to +48 of hIGFBP-5 cDNA as described in Methods section (Figure 9). Southern analysis and sequencing suggested that this insert contained over 3 kb of 5'-flanking sequence, all of the coding region, and part of the 3'-untranslated region of Exon 4. Southern analysis of both genomic DNA and the 50 kb cosmid insert demonstrated a 4.6 kb *Eco*RI restriction fragment which hybridized to the human IGFBP-5 cDNA and to an oligodeoxynucleotide probe corresponding to the 5'-untranslated region (Figure 9). This evidence suggested that the gene fragment was likely to contain the proximal 5'-flanking region and promoter. Therefore, this *Eco*RI fragment was subcloned into pGEM3Zf(-) for sequencing. The restriction map of the subclone is shown in Figure 10.

D. Structural Analysis of the hIGFBP-5 Gene

To verify that the 4.6 kb *Eco*RI genomic fragment contained regulatory regions of the hIGFBP-5 gene, the fragment was sequenced beginning from the first exon. The hIGFBP-5 sequence from position -461(relative to start of transcription) through the first exon was reported previously (Allander et al., 1994), and is identical to the sequence obtained.

Figure 7. PG increases IGFBP-5 gene transcription in U2 cells. U2 cells were treated for 1 h with 10 nM PG or vehicle control and nuclei were isolated. Transcription in 10^7 isolated nuclei per group was continued in the presence of [^{32}P]-UTP. ^{32}P-labeled hnRNA was hybridized to immobilized human ß-actin cDNA, human IGFBP-5 cDNA and pBluescript vector control DNA as described in Methods. The relative densities (mean of two replicates in each experiments) of ^{32}P-labeled hnRNA binding to target DNAs were measured by laser densitometry of the autoradiographs and are expressed as % of vehicle treated control.

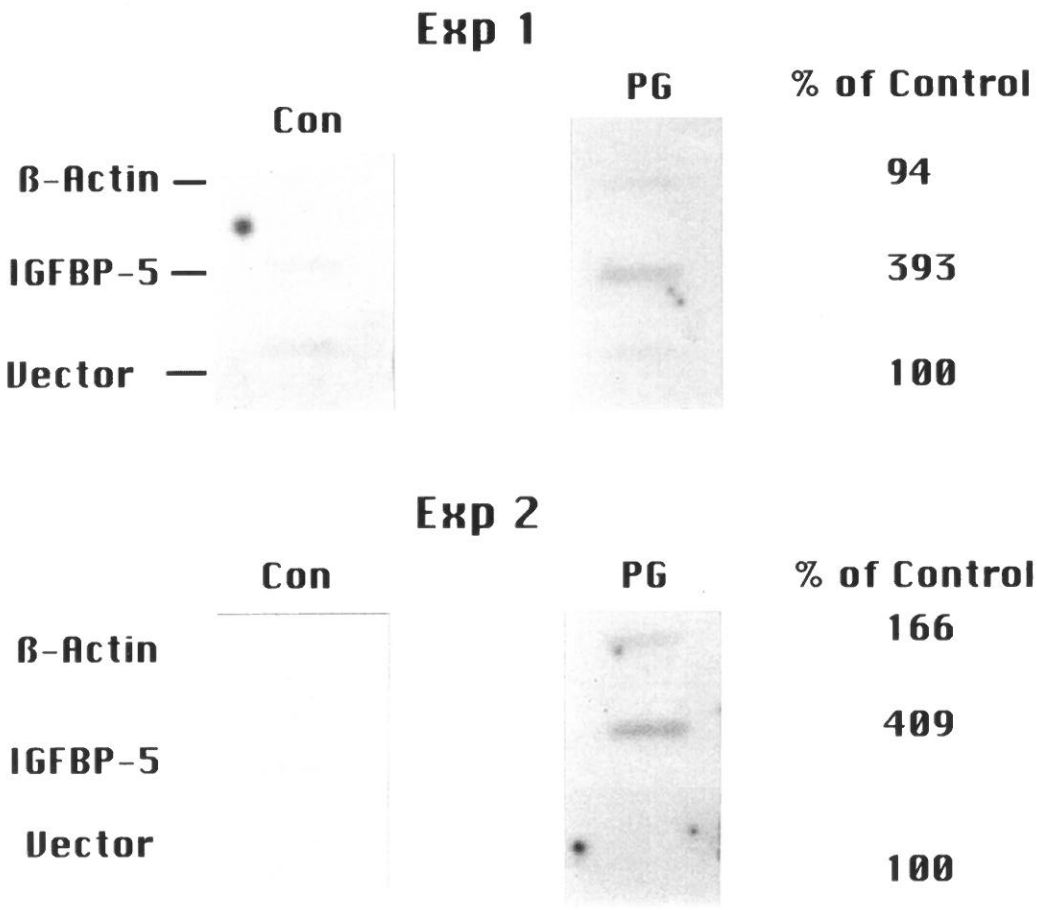

Exp 1
Con
PG
% of Control
ß-Actin
IGFBP-5
Vector
94
393
100
Exp 2
Con
PG
% of Control
ß-Actin
IGFBP-5
Vector
166
409
100

Figure 8. Diagram demonstrating isolation of the hIGFBP-5 gene. To isolate hIGFBP-5 clone, 4-6 x 10^5 cosmid clones from a human placental library were plated at high density and screened with a mixture of oligodeoxynucleotides corresponding to sequences in the 3'-untranslated (3'-UTR) region of the hIGFBP-5 cDNA sequence (Methods). Positive clones from the first step (top left panel) were replated and screened at high density. Positive clones from the second step (top right panel) were replated and screened again at low density. Positive clones from the third step (lower right panel) were replated and screened at low density (lower left panel) until all colonies on the plate hybridized strongly to the mixture of hIGFBP-5 3'-UTR oligodeoxynucleotides.

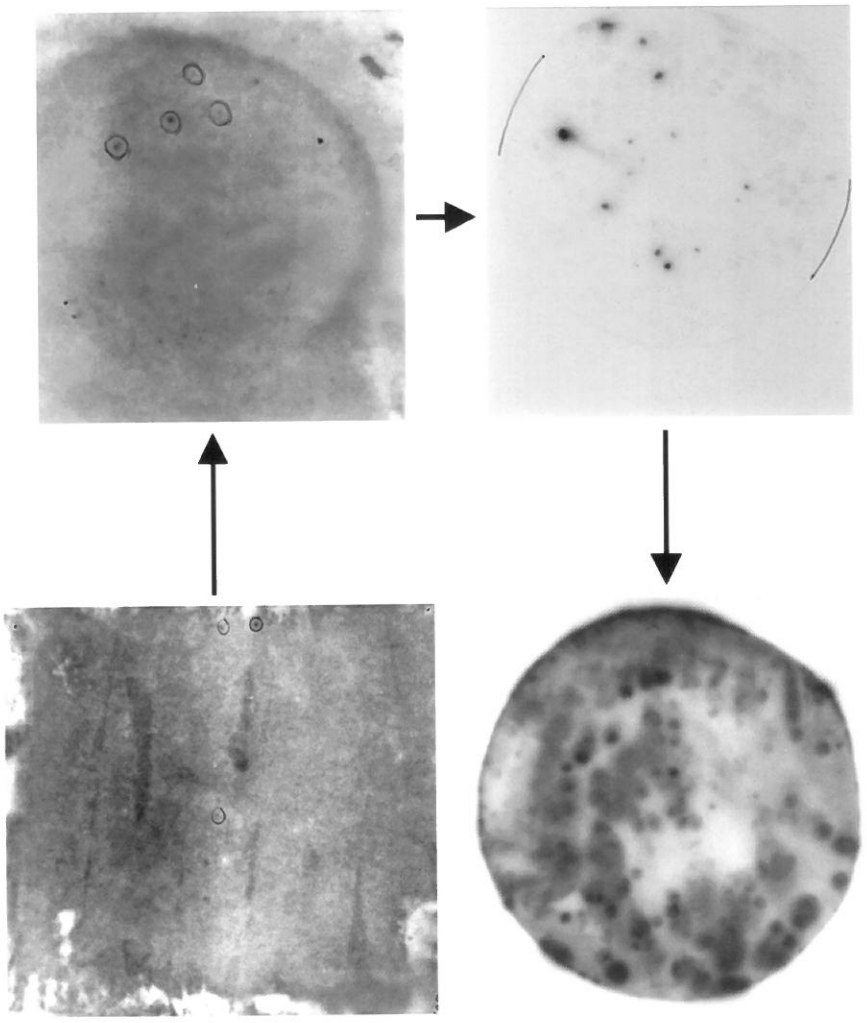


Figure 9. Restriction analysis of the hIGFBP-5 cosmid clone. Cosmid DNA from the positive clone was isolated, digested with restriction enzymes, and subjected to Southern Analysis using a ^{32}P-labeled hIGFBP-5 cDNA (left panel) or oligodeoxynucleotide corresponding to +28 to +48 of human IGFBP-5 cDNA (right panel) as probes. Names of restriction enzymes used are shown above each lane. The positions and sizes (bp) for Hinc III digested ϕx174, and Hind III digested λ DNA marker are indicated. The 4.6 kb *Eco*RI fragment which hybridized strongly to the ^{32}P-labeled hIGFBP-5 5'-UTR was subcloned into pGEM3Zf(-) and sequenced.

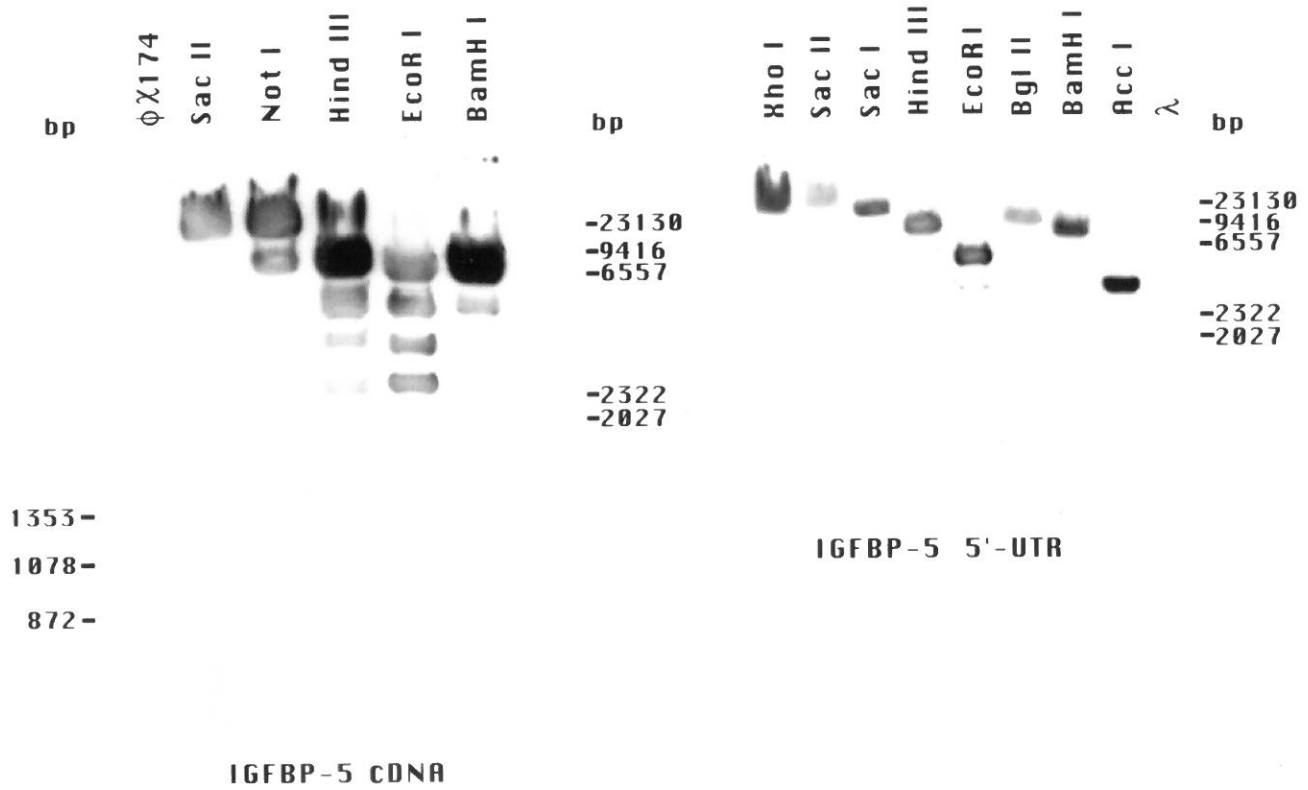
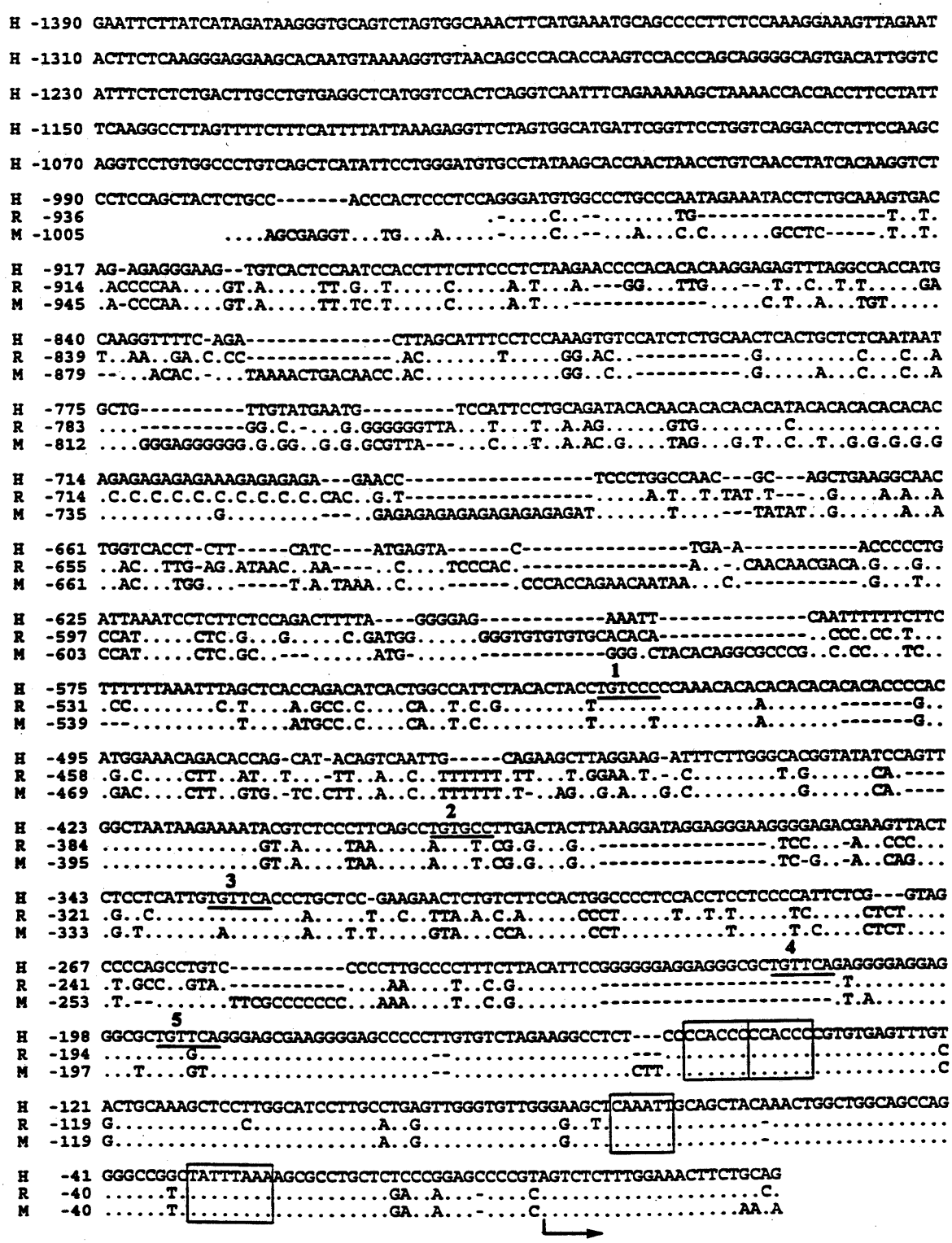

bp
φX174
Sac II
Not I
Hind III
EcoR I
BamH I
bp
-23130
-9416
-6557
-2322
-2027
1353-
1078-
872-
IGFBP-5 cDNA
Xho I
Sac II
Sac I
Hind III
EcoRI
Bgl II
BamH I
Acc I
λ
bp
-23130
-9416
-6557
-2322
-2027
IGFBP-5 5'-UTR

Figure 10. Sequences of human, rat, and mouse IGFBP-5 proximal promoter regions. The upper DNA strands of aligned DNA sequences of the human (H), rat (R), and mouse (M) IGFBP-5 gene 5'-flanking region are shown. Gaps in sequences are indicated by dash (--). Nucleotides in rat and mouse which are identical to the human sequences are indicated by a peroid (..). Position relative to transcription start site (arrow) and sequence of CAAT and TATA (boxes) are conserved in all three sequences. Putative progesterone response element half-sites (underlined) in the human IGFBP-5 sequence are homologous to the 3' half-site (position +2 to +7) of the optimally active PRE -7(A/G)GNACANNNTGTNC(C/T)+7 described in Lieberman, et al. (1993). Adjacent CACCC boxes are identical in the human, rat and mouse sequences.


H -1390 GAATTCTTATCATAGATAAGGGTGCAGTCTAGTGGCAAACTTCATGAAATGCAGCCCCTTCTCCAAAGGAAAGTTAGAAT
H -1310 ACTTCTCAAGGGAGGAAGCACAATGTAAAAGGTGTAACAGCCCACACCAAGTCCACCCAGCAGGGGCAGTGACATTGGTC
H -1230 ATTTCTCTCTGACTTGCCTGTGAGGCTCATGGTCCACTCAGGTCAATTTCAGAAAAAGCTAAAACCACCACCTTCCTATT
H -1150 TCAAGGCCTTAGTTTTCTTTCATTTTATTAAAGAGGTTCTAGTGGCATGATTCGGTTCCTGGTCAGGACCTCTTCCAAGC
H -1070 AGGTCCTGTGGCCCTGTCAGCTCATATTCCTGGGATGTGCCTATAAGCACCAACTAACCTGTCAACCTATCACAAGGTCT
H -990 CCTCCAGCTACTCTGCC-------ACCCACTCCCTCCAGGGATGTGGCCCTGCCCAATAGAAATACCTCTGCAAAGTGAC
R -936 .-....C..--.....TG----------T..T.
M -1005 ....AGCGAGGT...TG...A.....-....C..--...A...C.C......GCCTC-----.T..T.
H -917 AG-AGAGGGAAG--TGTCACTCCAATCCACCTTTCTTCCCTCTAAGAACCCCACACACAAGGAGAGTTTAGGCCACCATG
R -914 .ACCCCAA....GT.A.....TT.G..T.....C.....A.T...A.---GG...TTG...--.T..C..T.T.....GA
M -945 .A-CCCAA....GT.A.....TT.TC.T.....C.....A.T...----------.....C.T..A...TGT.....
H -840 CAAGGTTTTC-AGA----------CTTAGCATTTCCTCCAAAGTGTCCATCTCTGCAACTCACTGCTCTCAATAAT
R -839 T..AA..GA.C.CC----------.AC.....T.....GG.AC..----------.G.....C...C..A
M -879 --...ACAC.-...TAAAACTGACAACC.AC.....GG..C..----------.G.....A...C...C..A
H -775 GCTG----------TTGTATGAATG---------TCCATTCCTGCAGATACACAACACACACACATACACACACACAC
R -783 ....----------GG.C.-...G.GGGGGGTTA...T...T..A.AG.....GTG.....C.....
M -812 ....GGGAGGGGGG.G.GG..G.G.GCGTTA---...C...T..A.AC.G....TAG...G.T..C..T..G.G.G.G.G
H -714 AGAGAGAGAGAAAGAGAGAGA---GAACC----------TCCCTGGCCAAC---GC---AGCTGAAGGCAAC
R -714 .C.C.C.C.C.C.C.C.C.C.CAC..G.T----------.....A.T..T.TAT.T----.G....A.A..A
M -735 .....G.....---..GAGAGAGAGAGAGAGAGAT.....T....---TATAT..G.....A..A
H -661 TGGTCACCT-CTT-----CATC----ATGAGTA------C----------TGA-A----------ACCCCCTG
R -655 ..AC..TTG-AG.ATAAC..AA-----..C....TCCCAC.----------A..-.CAACAACGACA.G...G..
M -661 ..AC...TGG.....-----T.A.TAAA..C....-------.CCCACCAGAACAATAA...C.----------.G...T..
H -625 ATTAAATCCTCTTCTCCAGACTTTTA----GGGGAG----------AAATT----------CAATTTTTTCTTC
R -597 CCAT.....CTC.G...G.....C.GATGG.....GGGTGTGTGTGCACACA----------..CCC.CC.T...
M -603 CCAT.....CTC.GC..---.....ATG-.....----------GGG.CTACACAGGCGCCCG..C.CC...TC..
1
H -575 TTTTTTAAATTTAGCTCACCAGACATCACTGGCCATTCTACACTACCTGTCCCCCAAACACACACACACACACCCCAC
R -531 .CC.....C.T....A.GCC.C....CA..T.C.G.....T.....A.....-------G..
M -539 ---.....T....ATGCC.C....CA..T.C.....T.....T.....A.....-------G..
H -495 ATGGAAACAGACACCAG-CAT-ACAGTCAATTG-----CAGAAGCTTAGGAAG-ATTTCTTGGGCACGGTATATCCAGTT
R -458 .G.C....CTT..AT..T...-TT..A..C..TTTTTT.TT...T.GGAA.T.-.C.....T.G.....CA.----
M -469 .GAC....CTT..GTG.-TC.CTT..A..C..TTTTTT.T-..AG..G.A...G.C.....G.....CA.----
2
H -423 GGCTAATAAGAAAATACGTCTCCCTTCAGCCTGTGCCTTGACTACTTAAAGGATAGGAGGGAAGGGGAGACGAAGTTACT
R -384 .....GT.A....TAA.....A...T.CG.G...G..----------.TCC...-A..CCC...
M -395 .....GT.A....TAA.....A...T.CG.G...G..----------.TC-G..-A..CAG...
3
H -343 CTCCTCATTGTGTTCACCCTGCTCC-GAAGAACTCTGTCTTCCACTGGCCCCTCCACCTCCTCCCCATTCTCG---GTAG
R -321 .G..C.....A.....T..C..TTA.A.C.A.....CCCT.....T..T.T.....TC.....CTCT....
M -333 .G.T.....A.....A...T.T.....GTA...CCA.....CCT.....T.....T.C....CTCT....
4
H -267 CCCCAGCCTGTC----------CCCCTTGCCCCTTTCTTACATTCCGGGGGGAGGAGGGCGCTGTTCAGAGGGGAGGAG
R -241 .T.GCC..GTA.----------...AA....T..C.G.....----------.T.....
M -253 .T.--.....TTCGCCCCCCC...AAA....T..C.G.....----------.T.A.....
5
H -198 GGCGCTGTTCAGGGAGCGAAGGGGAGCCCCCTTGTGTCTAGAAGGCCTCT---CCCCACCCCCACCCCGTGTGAGTTTGT
R -194 .....G.....--.....---..|.....|.....|.....C
M -197 ...T....GT.....--.....CTT..|.....|.....|.....C
H -121 ACTGCAAAGCTCCTTGGCATCCTTGCCTGAGTTGGGTGTTGGGAAGCTCAAATTGCAGCTACAAACTGGCTGGCAGCCAG
R -119 G.....C.....A..G.....G..T.....|.....-.....
M -119 G.....A..G.....G....|.....|.....-.....
H -41 GGGCCGGCTATTTAAAAGCGCCTGCTCTCCCGGAGCCCCGTAGTCTCTTTGGAAACTTCTGCAG
R -40 .....T.....|.....GA..A...-....C.....C.
M -40 .....T.....|.....GA..A...-....C.....AA.A

The sequence from -1390 to -462 that is present in the *Eco*RI fragment, which was not present in the *Hind*III-*Pst*I fragment reported previously (Allander et al., 1994), has not been previously reported. The 5'-flanking region contains a TATA box and CAAT box, multiple progesterone response elements (PREs) and other putative response elements (Figure 10). A potential transcription initiation site (gtAgtc where A is the start of transcription) resides 33 nucleotides from the proximal TATA box . A putative second distal promoter complete with TATA (-1125 to -1119) and CAAT (-1187 to -1183) boxes was identified with a potential transcription initiation site (tcAgga) 31 nucleotides downstream of the TATA box (-1088 to -1083).

The transcription start site of the human IGFBP-5 promoter in breast cancer cells was identified previously as 33 nucleotides 3' from the TATA box corresponding to the proximal TATA box of the *EcoRI* fragment that we analyzed (Allander et al., 1994). To determine whether this site was also used to initiate transcription in human osteoblasts, primer extension analysis of U2 cell RNA was carried out. An end-labeled oligodeoxynucleotide corresponding to a location in the hIGFBP-5 5'-untranslated region, 65 nucleotides from the putative start site, was hybridized to 50 μg of U2 cell total RNA. The fragments that were produced by extension with reverse transcriptase were separated on an 8% polyacrylamide gel containing sequencing standards in adjacent lanes. This analysis revealed a single band 33 nucleotide from the TATA box, which is the same result obtained by Allander et al. No evidence was found supporting use of the putative distal promoter and transcription start site at position -1052 in human osteoblasts.

E. Structural Comparison of Human, Rat, and Mouse IGFBP-5 Promoters

As shown in Figure 10, the 1,390 bp 5'-flanking region of human IGFBP-5 was compared to the reported 936 bp of rat (Zhu et al., 1993) and 1065 bp of mouse (Kou et al., 1994) IGFBP-5 5'-flanking regions. The three IGFBP-5 proximal promoter sequences are 70% similar, with the greatest sequence similarity near the transcription initiation site and identical consensus sequences for the proximal promoter elements. The three sequences contain, in the same relative positions, identical TATA (-33) and CAAT (-73) boxes and a pair of ovelapping putative AP-2 binding sites (Becker et al., 1987; Faisst and Meyer, 1992) at position -148 and -139. Within the AP-2 binding sequences are two adjacent CACCC boxes (Bassel-Duby et al., 1992; Bassel-Duby et al., 1994; Chen et al., 1994; Kim et al., 1992; Miller and Beiker, 1993; Schule et al., 1988; Wang et al., 1993).

The consensus glucocorticoid/progesterone response element (GRE/PRE) is composed of two hexameric half-sites arranged as a palindrome with a 3-base pair spacer, GGTACANNNTGTTCT (Lieberman et al., 1993). A computer analysis of 1392 bp of the hIGFBP-5 gene 5'-flanking region revealed the absence of consensus GRE/PRE sequences. However the hIGFBP-5 promoter contains five putative GRE/PRE half sites at position -528, -392, -333, -215 and -193, which have strong sequence similarity to the hexanucleotide motif 5'-TGTTCT-3' of the MMTV promoter and 5'-TGTTCA-3' of the uteroglobin gene promoter (Bailly et al., 1986; Beato et al., 1989). Although GR and PR do not bind to GRE/PRE half-sites as effectively as to the palindromic GRE/PRE

consensus sequence, clusters of GRE/PRE half-site sequences in the MMTV, uteroglobin and other promoters are functional. GR and PR bind to the half-sites and effectively mediate ligand dependent transactivation through synergistic interactions between the mutiple half-sites (Bailly et al., 1986; Beato et al., 1989; Lieberman et al., 1993).

PRE half-site sequences are also present in the rat and mouse IGFBP-5 promoters (Figure 10) and in the rat IGFBP-1 promoter (Goswami et al., 1994), supporting the hypothesis that these sequences may be functionally significant. The rat IGFBP-5 promoter contains, at the same relative position as the human IGFBP-5 promoter, two PRE hexameric half-site sequences which are identical and one which is similar (five of six nucleotides) to the human. The mouse IGFBP-5 promoter contains two PRE half-site sequences which match the corresponding human PREs in five of six nucleotide positions. The rat IGFBP-1 promoter contains a single 12 bp imperfect GRE/PRE sequence which binds to the glucocorticoid receptor protein in DNA footprinting experiments (Goswami et al., 1994). Nine of the 12 nucleotides that comprise the 5'- and 3'-half-sites of the rat IGFBP-1 GRE/PRE sequence (position -7 to -5 and position +2 to +7) are identical to human IGFBP-5 PRE-4 and PRE-5 sequences at -215 and -193 (Figure 10).

F. Functional Analysis of the hIGFBP-5 Promoter

To determine whether the putative hIGFBP-5 promoter functions in human osteoblasts, CAT reporter constructs containing a 776 bp *Pst*I fragment (pCAT753) and a

484 bp *Hind*III/*Pst*I fragment (pCAT461) were prepared. Both constructs contain the transcriptional start site, 23 bp of 5'-untranslated region, and the putative overlapping AP-2/CACCC sequences. The pCAT753 construct contains five putative PRE half-sites and pCAT461 contains four. Subconfluent U2 cells were transiently transfected with the sense reporter construct, antisense construct, or the pJFCAT1 promoterless control vector. When U2 cells were transfected with pCAT753 or pCAT461 sense construct and incubated for 48 h, CAT activity increased to 620 and 730% respectively, of CAT activity in cells transfected with pJFCAT1 promoterless construct (Figure 11). CAT activity from antisense construct was the same as pJFCAT1. These results suggest that the sequence from -461 to +23 contains a functional promoter.

To investigate whether the putative TATA box at position -1052 functions in human osteoblasts, a CAT reporter construct containing a 643 bp *Pst* I fragment from positions-1390 to -747 (pCAT643) was prepared. Subconfluent U2 cells were transiently transfected with sense reporter construct, antisense reporter construct or the pJFCAT1 promoterless control. No increase in CAT activity was observed when cells were transfected with pCAT643 compared to pJFCAT1 promoterless control. Therefore, these results suggest that the TATA box at position -1052 does not function in human osteoblasts.

G. PG Induction of the hIGFBP-5 Promoter is PR Isoform Specific

To evaluate whether the 5'-flanking region of the hIGFBP-5 gene mediates PG induction of transcription, PG induction of CAT expression from pCAT753 and pCAT461 was determined. To reduce basal steroid exposure and optimize responsiveness to PG, U2 cells were maintained for 72 h in phenol red-free DMEM supplemented with 5% charcoal-dextran treated fetal bovine serum (CD-FBS) before transfection. Cells were co-transfected with pCAT753 (or pCAT461) and pCMV-ß-gal, then incubated 48 h in phenol red-free DMEM supplemented with 2% CD-FBS in the absence or presence of 10 nM PG.

In several replicate experiments with pCAT753, 10 nM PG increased CAT activity to 140% of control ($p<0.01$, Figure 12). PG activated transcription of pCAT461 to the same degree as transcription of pCAT753, suggesting that the PRE at position -528 and other sequences distal to -461 were not essential for PG stimulation of hIGFBP-5 promoter activity. The small but significant effect of PG on IGFBP-5 promoter activity suggested that PG might induce IGFBP-5 transcription by a PR-dependent mechanism utilizing the low levels of endogenous PRs in the U2 cells. Alternatively, PG might induce IGFBP-5 transcription by a PR-independent mechanism. To determine whether PG induction of IGFBP-5 transcription involves PR's, we cotransfected U2 cells with PR expression vectors.

PRs are expressed in various cells as two different isoforms which are transcribed from separate promoters in the PR gene (Tora et al., 1988). The B-receptor isoform

(PR-B) is 933 amino acids in length and the A-receptor isoform (PR-A) lacks 164 amino acids at the N-terminus. To determine whether PG induction of IGFBP-5 transcription is mediated by PRs and to define the specificity of the PR isoform, U2 cells were transiently transfected with either human PR-A or PR-B expression vectors, phPR-A and phPR-B (Vegato et al., 1993), along with pCAT753 and pCMV-ß-gal. When U2 cells were co-transfected with expression vector phPR-A , along with pCMV-ß-gal, PG reproducibly increased hIGFBP-5 promoter activity to 200-300 % of control, significantly higher than in cells transfected with vector alone (Figure 13). The same result was obtained after treatment of co-transfected cells with promegestone (R5020), a non-metabolizable progestin analog. The optimal DNA concentration for phPR-A was 2 μg per 60 mm dish. In cells transfected with phPR-B DNA (1 to 5 μg per 60 mm dish), PG did not increase IGFBP-5 promoter activity more than cells transfected with vector alone. Overexpression of PR-B, therefore, did not increase hIGFBP-5 promoter activity (Figure 13). In addition, when equal amounts of phPR-A and phPR-B were mixed and co-transfected with pCAT753 and pCMV-ß-gal, PG increased hIGFBP-5 promoter to the same extent as overexpressing PR-A alone, 200-300 % of control. This result suggests that PR-A acts as a dominant positive regulator of PG induced hIGFBP-5 gene transcription.

To ensure that functional PR's were expressed from phPR-A and particularly from phPR-B in U2 cells, cells were transiently transfected with either phPR-A or phPR-B together with the MMTV promoter-reporter construct pMSG-CAT and were subsequently treated with PG or vehicle control.

Figure 11. Demonstration of hIGFBP-5 promoter activity. DNA fragments containing 461 (pCAT461) or 753 bp (pCAT753) of hIGFBP-5 5'-flanking region were used to drive expression of the CAT reporter gene and are compared to promoterless control, pJFCAT1. The promoter-reporter constructs were transiently transfected into U2 cells and incubated in DMEM supplemented with 10% CS as described in Methods. CAT activity was measured 48 h after transfection, normalized to ß-galactosidase activity from respective groups, and expressed as % of control (cells transfected with pJFCAT1). Bar graphs represent mean CAT activity ± SD. In both pCAT461 and pCAT753 groups, CAT activities were significantly greater than basal ($p<0.001$), as determined by ANOVA.

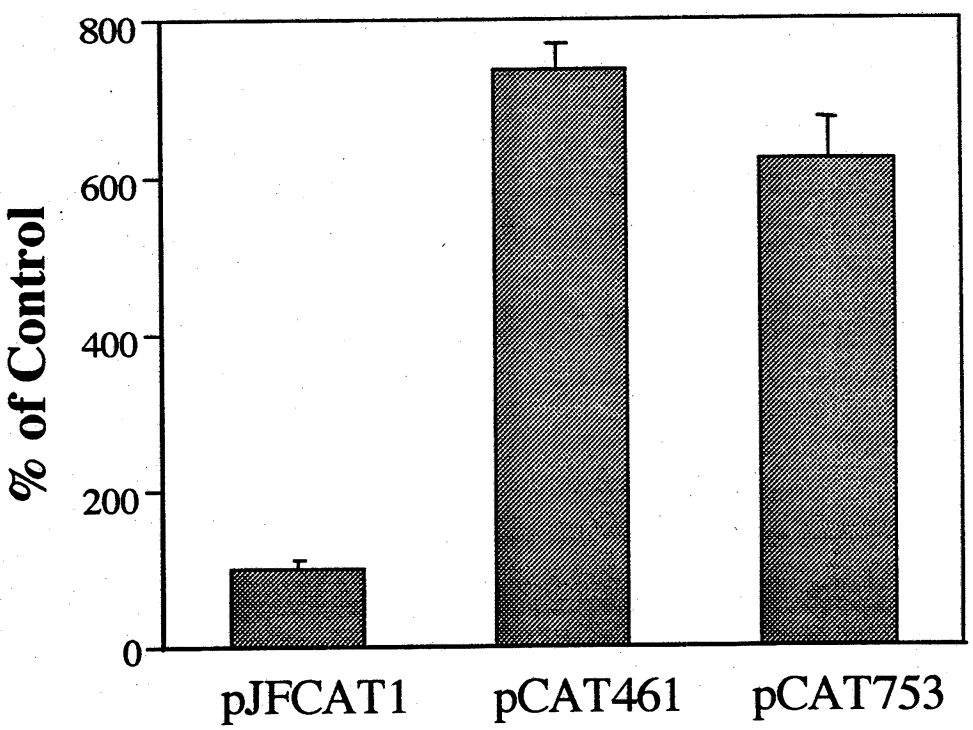

800
600
400
200
0
% of Control
pJFCAT1
pCAT461
pCAT753

Figure 12. PG increases IGFBP-5 promoter activity. The promoter-CAT reporter construct, pCAT753, was transiently transfected, along with pCMVß-gal, into U2 cells and incubated in phenol-red free DMEM supplemented with 2% CD-FBS in the absence or in the presence of 10 nM PG as described in Methods. CAT activity was normalized to ß-galactosidase activity from respective groups, and expressed as % of control (cells transfected with pJFCAT1), mean ± SD of five experiments. PG treatment significantly increased CAT activity ($p<.01$), as determined by Student's paired t-Test and by Kruskal-Wallis non-parametric test.

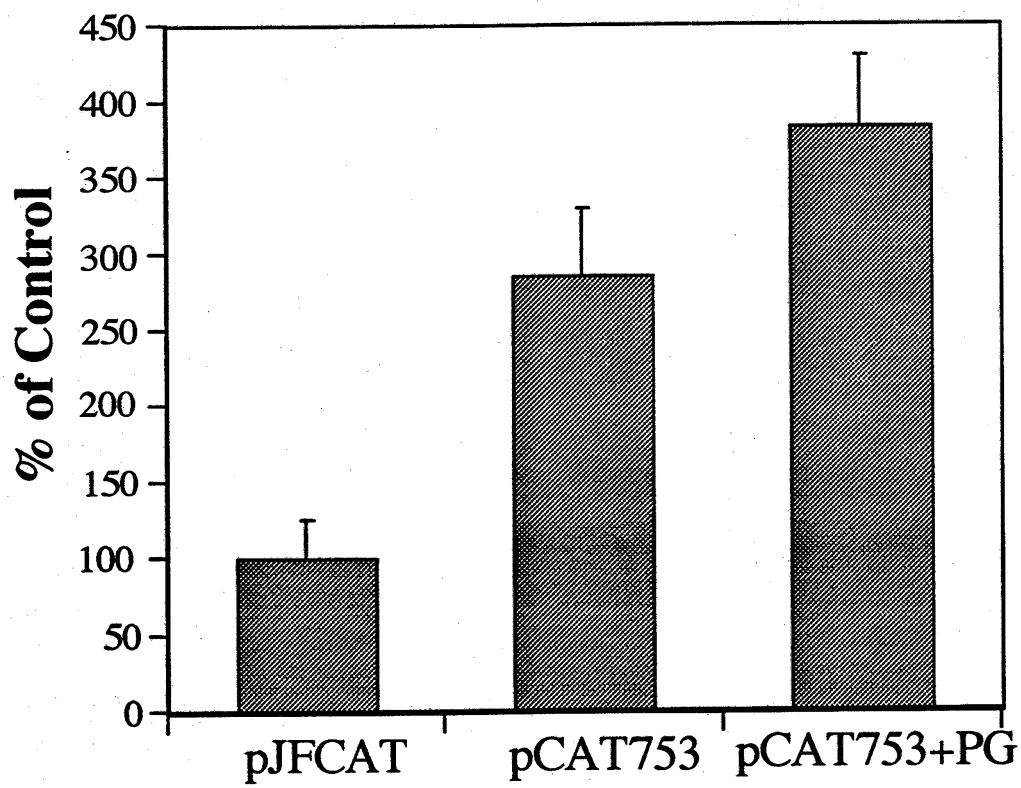

% of Control
450
400
350
300
250
200
150
100
50
0
pJFCAT
pCAT753
pCAT753+PG

Figure 13. PG induction of IGFBP-5 promoter activity is receptor type specific. U2 cells were transiently transfected with 3 μg of pCAT753 plus 2 μg of PR expression vector alone (without PR coding sequence), phPR-A or phPR-B. After transfection, cells were treated with 10 nM PG for 24 h. CAT activity was normalized to ß-galactosidase activity and expressed as % of control (cells transfected with CAT reporter construct plus vector alone, treated with solvent control). Values are mean ± SD of at least three independent experiments.

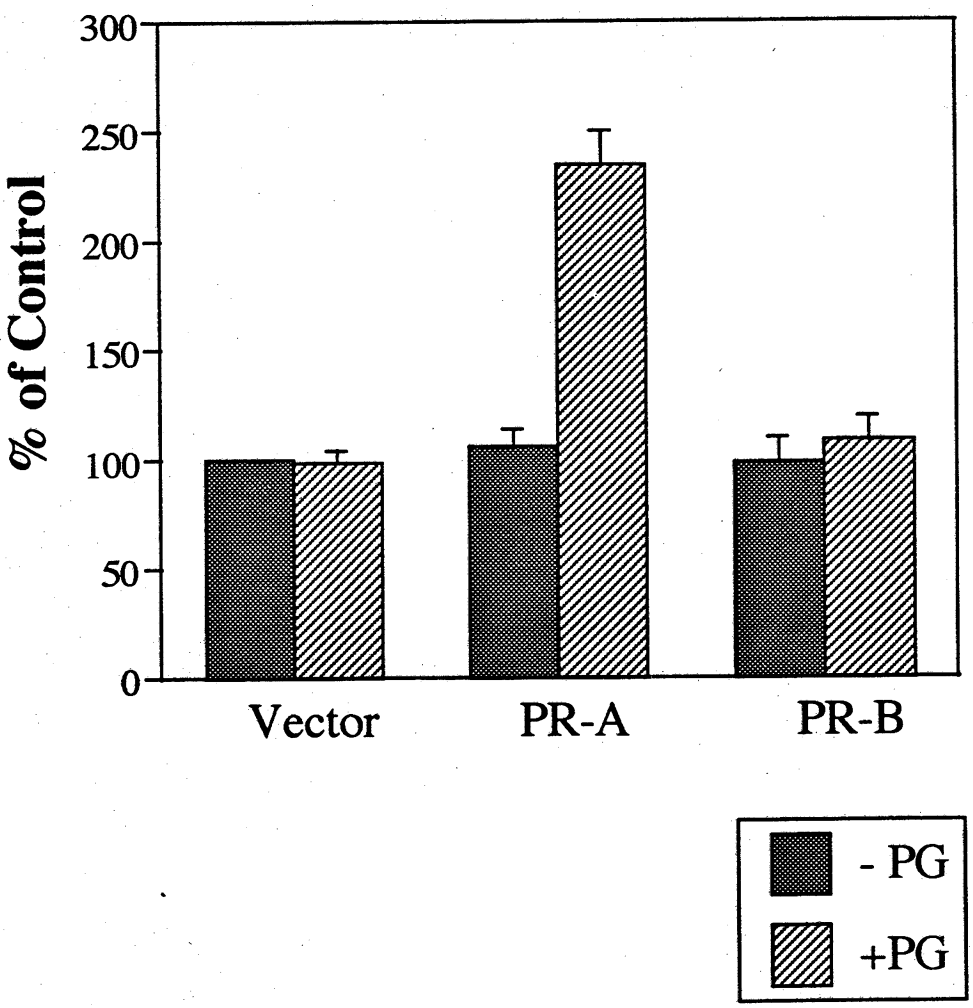
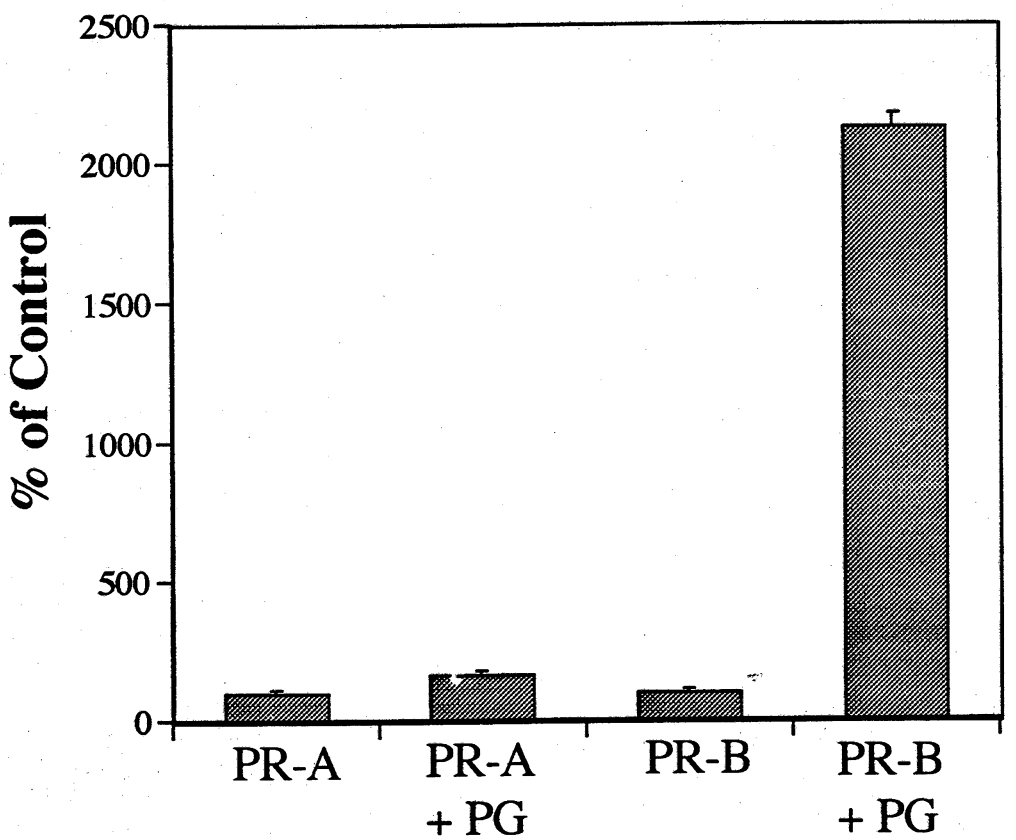

300
250
200
150
100
50
0
% of Control
Vector
PR-A
PR-B
- PG
+PG

Figure 14. Functional PR isoforms are expressed from expression vectors in U2 cells. U2 cells were transiently transfected with 3 μg of pMSG-CAT, which contains the mouse mammary tumor virus long terminal repeat, plus 2 μg of PR-A or PR-B expression vector ,phPR-A or phPR-B, respectively. After transfection, cells were treated with 10 nM PG for 24 h. CAT activity was normalized to ß-galactosidase activity and expressed as % of respective control groups (cells transfected with CAT reporter construct plus phPR-A or phPR-B, treated with solvent control). Values are mean ± SD of at least three independent experiments.


% of Control
2500
2000
1500
1000
500
0
PR-A
PR-A + PG
PR-B
PR-B + PG

This construct contains a functional PRE from the MMTV long terminal repeat. Both phPR-A and phPR-B conferred PG inducibility to the MMTV promoter to 170% and 2000% of control respectively (Figure 14), demonstrating that PR-B was effectively expressed in U2 cells. This result was consistent with a previous report (Tora et al., 1988) demonstrating that PR-B was a more efficient activator of the MMTV promoter than was PR-A. In contrast to the relative effects on the MMTV promoter, PG reproducibly increased hIGFBP-5 promoter activity to 200-300% of solvent treated control in U2 cells overexpressing PR-A (Figure 13) but did not increase IGFBP-5 promoter activity in cells overexpressing PR-B. These data support the conclusion that PG induction of hIGFBP-5 promoter activity is mediated by PR-A but not PR-B.

H. Identification of hIGFBP-5 Promoter Sequences which Confer PG Responsiveness-the Progesterone Dependent Response Element (PDRE)

As a first step to determine whether the putative PRE half-sites identified in the hIGFBP-5 proximal promoter mediate effects of PG on hIGFBP-5 transcription, a series of 5'-deletions were made which contained progressively fewer PRE half-site sequences. Constructs contained 753, 461, 345, 325, 252, 162, and 124 bp of the hIGFBP-5 5'-flanking region with 5, 4, 3, 2 , 2 and 0 putative PRE half-sites, respectively (Figure 3). Basal promoter activity of each construct determined in cells maintained in DMEM plus 10% calf serum was 300% to 2000% of the promoterless control group (Figure 15). PG induction of activity from each hIGFBP-5 promoter deletion construct was

determined in cells pre-cultured in DMEM + 5% CD-FBS, cotransfected with phPR-A, pCMV-β-gal and the deletion construct, then changed to DMEM + 2% CD-FBS with solvent or 10 nM PG. Basal promoter activities in these conditions were lower than when cells were in 10% CS. In multiple experiments PG reproducibly increased promoter activity from all deletion constructs from pCAT753 to pCAT162 by 200-300% of the respective solvent treated controls (Figure 16). However, PG failed to increase CAT activity in cells transfected with pCAT124 which contained only the CAAT and TATA boxes, although pCAT124 and pCAT162 had similar levels of basal promoter activity. These data suggest that (1) none of the putative PRE half-sites were required for ligand-dependent transactivation of the hIGFBP-5 promoter and (2) the element between -162 and -124, which contains two tandem CACCC box sequences (Chen et al., 1994; Direks et al., 1983; Kim et al., 1992), may confer PG responsiveness to the hIGFBP-5 promoter.

The CACCC box sequence is an important promoter element required for efficient and accurate gene expression in the ß-globin gene (Direks et al., 1983). In addition, the CACCC box can interact with several transcription factors including the retinoblasotoma control element (RCE) binding protein, myocyte nuclear factor, and other CACCC-binding proteins (Bassel-Duby et al., 1994; Faisst and Meyer, 1992; Miller and Beiker, 1993; Wang et al., 1993). CACCC-binding proteins can either bind directly to the CACCC box or interact with other transcription factors and bind as a complex (Schule et al., 1988).

To determine whether the CACCC sequences between position -162 and -124 are the critical elements that are required for PG stimulation of hIGFBP-5 promoter activity, both (pCAT162mut), distal (pCAT162mDi), or proximal (pCAT162mPr) CACCC sequences in pCAT162 were mutated from CCCACCC to AAAACCC. PG induction determined in cells pre-cultured in DMEM + 5% CD-FBS, co-transfected with phPR-A, pCMV-ß-gal, and pCAT162 mutant constructs or wild type pCAT162, then changed medium to DMEM + 2% CD-FBS with solvent or 10 nM PG. In multiple experiments, PG increased pCAT162 and pCAT162mDi promoter activities but failed to increase pCAT162mut and pCAT162mPr promoter activities (Figure 17). Thus, mutation of the proximal CACCC box at position -139 abolished PG transactivation hIGFBP-5 promoter activity. These data provide strong evidence that the proximal CACCC sequence at position -139 is required for PG dependent transactivation of hIGFBP-5 promoter activity.

I. CACCC Binding Sequence in the hIGFBP-5 Promoter

To determine whether U2 cell nuclear proteins interact with the PG responsive hIGFBP-5 promoter sequence -124 to -162, electrophoretic mobility shift assays (EMSA) were performed. Double stranded synthetic oligodeoxynucleotides were prepared corresponding to the wild type hIGFBP-5 promoter sequence from -155 to -128, termed the hIGFBP-5 Progesterone Dependent Responsive Element (BP5-PDRE). BP5-PDRE oligodeoxynucleotide was end-labeled and incubated with 3 μg of nuclear extract from

U2 cells that had been incubated in phenol red free DMEM supplemented with 2% CD-FBS for 48 h. Analysis by EMSA resulted in a reproducible set of shifted bands (Figure 18A). To determine the specificity of this nuclear factor binding, nuclear extract proteins were incubated with labeled BP5-PDRE in the presence of excess concentrations of the unlabeled oligodeoxynucleotide BP5-PDRE or oligodeoxynucleotides containing functional CACCC box core motif sequences from other promoters (cold competition): RCE sequences from the TGF-ß and c-fos promoters, and the MNF binding site sequence from the myoglobin promoter. Cold competition was also assessed with oligodeoxynucleotides corresponding to the consensus AP-2 binding site sequence from the metallothionein-IIA promoter (Williams et al., 1988) and the consensus Sp1 binding site sequence from Simian virus 40 (Briggs et al., 1986) because the transcription factor Sp1 had been shown to bind to an RCE (Chen et al., 1994), and a CACCC box has been shown to function as an alternate AP-2 response element (Faisst and Meyer, 1992). The oligodeoxynucleotides were all approximately the same length. The sequence, promoter source, and reference of each oligodeoxynucleotides are described in Table 1. As shown in Figure 18A, a 50 fold molar excess of unlabeled BP5-PDRE specifically competed with labeled BP5-PDRE for binding to U2 cell nuclear protein. At 50 fold excess molar ratio of unlabeled DNA to labeled BP5-PDRE DNA, consensus AP-2, Sp1, and RCE sequences did not compete as effectively with labeled BP5-PDRE for binding. However, 25, 50 and 100 fold molar excess of the MNF binding sequence did effectively compete with labeled BP5-PDRE for nuclear protein binding (Figure 18B). In comparison, 50 and

100 fold molar excess of consensus AP-2 did not effectively compete for binding (Figure 19). These results suggest that nuclear protein(s) which bound to the BP5-PDRE DNA sequence were not transcription factors AP-2, Sp1, or RCE binding proteins but could be factors related to MNF. MNF is a novel winged-helix transcription factor that binds to a CACCC motif-containing response element in the human myoglobin promoter (Bassel-Duby et al., 1992; Bassel-Duby et al., 1994; Faisst and Meyer, 1992). The myoblobin MNF binding site contains a single CCCCACCCC sequence which is identical to the CCCCACCCC sequence in the hIGFBP-5 promoter that confers PG responsiveness.

To further define DNA sequences in the BP5-PDRE which were required for binding of nuclear proteins, both CCCACCC sequences between -147 to -139 were mutated to AAAACCC (BP5-PDREm1, Table 1). This is the same mutation that eliminated PG responsiveness in the hIGFBP-5 promoter-reporter construct, pCAT162mut. As shown in Figure 18B, BP5-PDREm1 did not compete with labeled BP5-PDRE oligodeoxynucleotides, supporting the conclusion that CACCC sequences in the BP5-PDRE sequence are required for nuclear factor binding. To determine if one or both tandem CACCC boxes in the hIGFBP-5 promoter bound to nuclear factors, the CACCC sequences were individually mutated from CCCACCC to CCAACCC. Mutation of both distal and the proximal (BP5-PDREm2, Table 1) or mutation of only the proximal CACCC sequence (BP5-PDREm4, Table 1) eliminated competition with wild type labeled BP5-PDRE for nuclear factor binding (Figure 18B). However, mutation of

only the distal CACCC sequence (BP5-PDREm3, Table 1) did not eliminate the ability of the oligodeoxynucleotide to compete with labeled BP5-PDRE for nuclear factor binding, suggesting that the distal CACCC sequence is not essential for forming the DNA-protein complexes observed by EMSA. This finding is consistent with the mutation of pCAT162 which demonstrated that only the proximal CACCC box at position -139 was required for PG transactivation of hIGFBP-5 gene transcription. Together, these data strongly suggest that the proximal CACCC box at position -139 is essential for the binding of U2 cell nuclear protein(s) and PG transactivation of hIGFBP-5 gene transcription.

J. PR-A Interaction with Nuclear BP5-PDRE Binding Protein(s)

Deletion of region -162 to -124 and mutation of the CACCC sequences within this region abolished PG responsiveness of the hIGFBP-5 proximal promoter. In addition, the BP5-PDRE oligodeoxynucleotide corresponding to this region of the promoter bound to U2 cell nuclear protein(s). These results suggest that PR-A could either directly bind to the BP5-PDRE or interact with nuclear protein(s) which bind to the BP5-PDRE. To test these possibilities, EMSAs were carried out to examine the binding of purified baculovirus-expressed human PR-A to the BP5-PDRE. The functionality of the PR-A preparation was first tested by EMSA with a consensus progesterone response element oligodeoxynucleotide (MMTV-PRE) from the mouse mammary tumor virus promoter (El-Ashry et al., 1989) and the PR monoclonal antibody, AB52 which binds both PR-A and PR-B (Onate et al., 1994). Labeled MMTV-PRE was incubated with

purified PR-A, with U2 nuclear extract, with nuclear extract plus purified PR-A or with nuclear extract plus purified PR-A and the PR monoclonal antibody. When U2 nuclear extract alone was incubated with labeled MMTV-PRE, a diffuse nonspecific binding complex was observed as a mobility-shift band which could not be abrogated or supershifted by incubating with PR antibody. Purified PR-A alone did not bind to labeled MMTV-PRE, as observed previously (Onate et al., 1994). However, in the presence of 1 μg of nuclear extract, purified PR-A bound to labeled MMTV-PRE and produced a strong mobility shift band which was supershifted with the PR antibody (Figure 20A). These results are consistent with the previous observation (Onate et al., 1994) that accessory proteins in the nuclear extract are required for PR to form functional receptor complexes which bind to the PRE. Similar results were obtained when purified PR-B was used instead of PR-A.

When PR-A protein in the absence of nuclear extract was tested for binding to the BP5-PDRE oligodeoxynucleotide, it did not cause a band shift, which is similar to the result obtained with the MMTV-PRE (Figure 20B). Incubation of labeled BP5-PDRE with 5 μg of U2 cell nuclear protein alone produced multiple shifted bands as shown in Figure 18. When increasing amounts of purified PR-A were pre-incubated with nuclear extract and then mixed with labeled BP5-PDRE, the intensity of bands 1 and 3 progressively decreased while the intensity of band 4 progressively increased (Figure 20B). In contrast, when increasing amounts of purified baculovirus expressed PR-B were incubated with 5 μg of nuclear extract protein and labeled BP5-PDRE, the

gelshift pattern was not altered (Figure 21). This result is consistent with the previous finding that overexpression of PR-A but not PR-B increased the magnitude of PG dependent transactivation of the hIGFBP-5 promoter.

Addition of PR antibody together with PR-A and nuclear proteins did not alter the gel shift pattern of labeled BP5-PDRE (Figure 20B), suggesting that either PR-A did not participate in direct binding to BP5-PDRE or that the PR-A epitope to which the PR-monoclonal antibody binds was masked by the nuclear proteins. As an alternative approach to test whether PR-A bound directly to the BP5-PDRE sequence, the binding of nuclear proteins to labeled BP5-PDRE was competed with excess amount of unlabeled consensus MMTV-PRE DNA, which efficiently binds to PR-A in the presence of nuclear extract (Figure 20A). If PR-A binds directly to BP5-PDRE, addition of excess unlabeled MMTV-PRE DNA should efficiently compete with labeled BP5-PDRE in binding to PR-A , reduce PR-A binding to BP5-PDRE and alter the band shift pattern. However, addition of a 1000 fold molar excess of unlabeled MMTV-PRE to PR-A, nuclear proteins, and labeled BP5-PDRE did not alter the gel shift pattern, further supporting the conclusion that PR-A did not bind directly to BP5-PDRE.

To determine whether PR-A effects on U2 cell nuclear proteins were specific to the proteins which bind to BP5-PDRE, we compared the effects of PR-A on gel shift patterns of BP5-PDRE with effects on gel shift patterns of other responsive elements (Sp1 and TGF-ß RCE). Labeled BP5-PDRE and labeled Sp1 oligodeoxynucleotides were each incubated with 5 μg of U2 nuclear extract in the presence of 0, 6, or 8 fmol of

purified PR-A. Purified PR-A interacted with BP5-PDRE binding proteins and changed the mobility shift pattern of BP5-PDRE (Figure 21), but PR-A did not affect the mobility shift pattern resulting from the binding of Sp1 and other nuclear proteins to the labeled Sp1 oligodeoxynucleotide. Similarly, PR-A did not affect the mobility shift pattern resulting from the binding of TGFß-RCE binding proteins to labeled TGFß-RCE oligodeoxynucleotide. Thus among the response elements that were analyzed, the interaction of PR-A with U2 cell nuclear factors is specific to BP5-PDRE binding protein(s)

Figure 15. Basal promoter activity of hIGFBP-5 promoter-reporter deletion constructs. In this representative experiment, promoter-reporter constructs were transiently transfected into U2 cells and incubated in DMEM supplemented with 10% CS as described in Methods. CAT activity was normalized to ß-galactosidase activity from respective groups and express as % of basal activity (cells transfected with pJFCAT1), mean ± SD, n = 4 replicates.

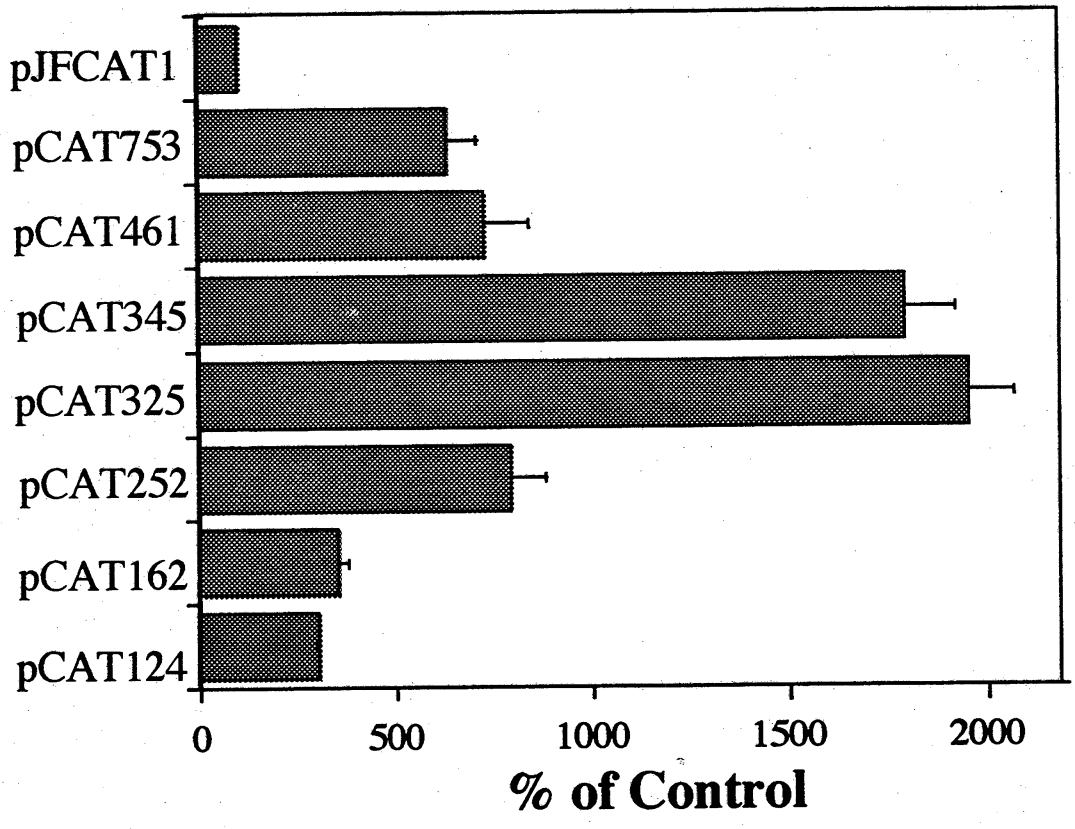

pJFCAT1
pCAT753
pCAT461
pCAT345
pCAT325
pCAT252
pCAT162
pCAT124
0
500
1000
1500
2000
% of Control

Figure 16. PG induction of promoter activity in hIGFBP-5 promoter-reporter deletion constructs. The promoter-reporter constructs shown in Figure 3 were transiently transfected into U2 cells along with phPR-A and pCMVß-gal as described in Methods, and incubated in 2% CD-FBS. CAT activities were normalized to ß-galactosidase activities and expressed as % of respective control groups (cells transfected with phPR-A and promoter-reporter construct incubated without PG), mean ± SD of at least three independent experiments for each construct. There was no significant difference in fold-induction of CAT expression by PG in groups pCAT753-pCAT162, as determined by ANOVA. PG induction of CAT expression in the pCAT124 group was significantly lower than in all other groups ($p<.001$).

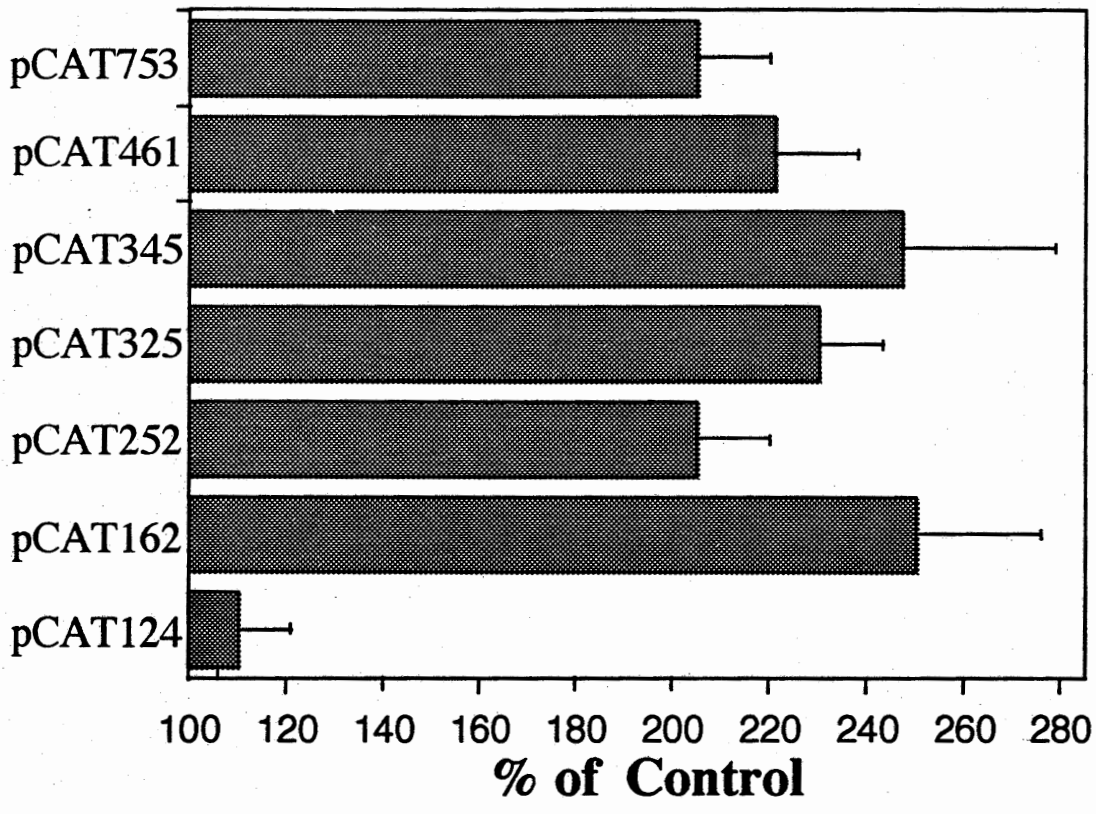

pCAT753
pCAT461
pCAT345
pCAT325
pCAT252
pCAT162
pCAT124
100 120 140 160 180 200 220 240 260 280
% of Control

Figure 17. Mutation of CACCC boxes eliminates PG induction of hIGFBP-5 promoter activity. U2 cells were transfected with phPR-A, pCMVß-gal and promoter-reporter constructs: pCAT162, pCAT162mut, pCAT162mDi, pCAT162mPr, or pCAT124. Both CACCC box sequences at position -147 to -139 were mutated from CCCACCCCCACCCC to AAAACCAAAACCCC in pCAT162mut. Only the distal CACCC box was mutated to AAAACCCCCACCCC in pCAT162mDi and only the proximal CACCC box was mutated to CCCACCAAAACCCC in pCAT162mPr. Cells were incubated in 2% CD-FBS in the presence or absence of 10 nM PG. CAT activities were normalized to ß-galactosidase activities and expressed as % of respective control group (transfected cells incubated in the absence of PG), mean ± SD of three independent experiments. PG significantly increased CAT activities in pCAT162 and pCAT162mDi transfected cells ($p<.001$) but did not significantly affect CAT activities in pCAT162mut, pCAT162mPr or pCAT124 transfected cells.

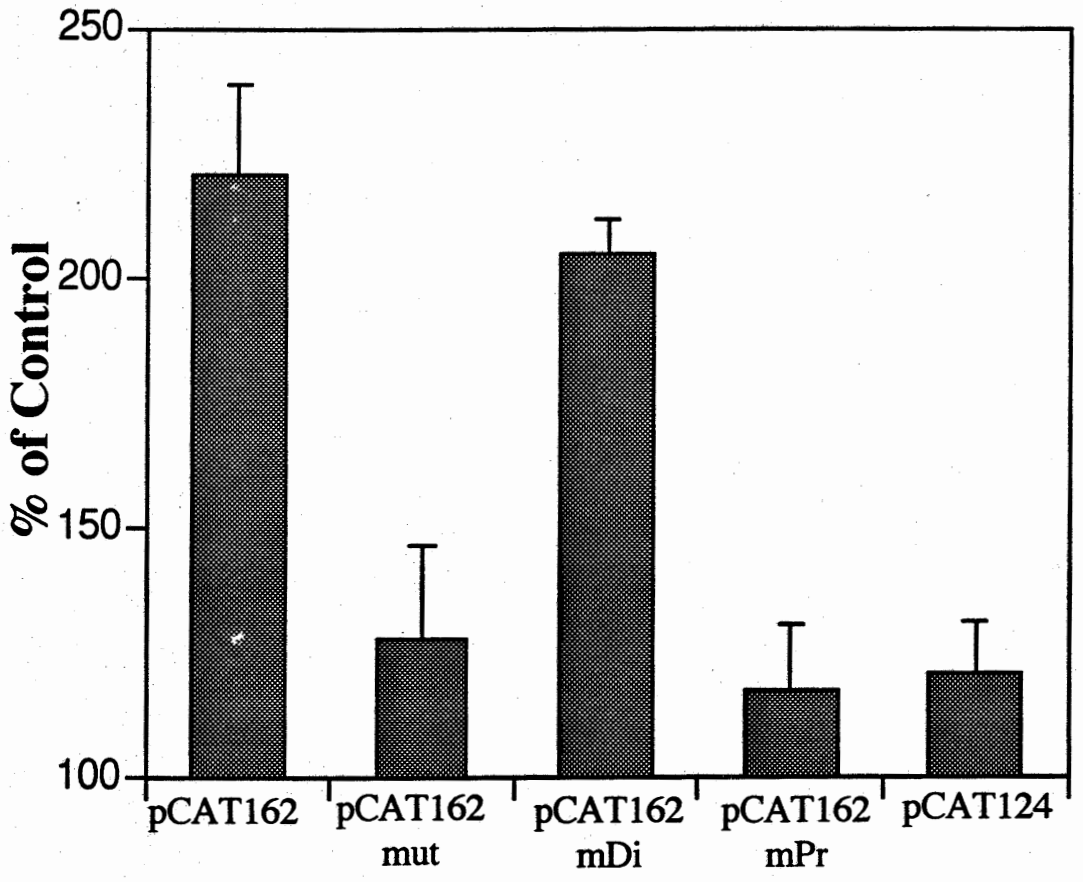

250
200
150
100
% of Control
pCAT162
pCAT162 mut
pCAT162 mDi
pCAT162 mPr
pCAT124

Table 1 Sequence of oligodeoxynucleotides used as probes and/or competitors in EMSA experiments

Oligonucleotide	Sequence	Gene
BP5-PDRE	5′-CCTCTCCCCACCCCCACCCCGTGTG-3′ 3′-GGAGAGGGGTGGGGGTGGGGCACAC-5′	IGFBP-5
BP5-PDREm1	5′-CCTCTCaaaACCaaaACCCCGTGTG-3′ 3′-GGAGAGtttTGGtttTGGGGCACAC-5′	Mutated IGFBP-5
BP5-PDREm2	5′-CCTCTCCCaACCCCaACCCCGTGTG-3′ 3′-GGAGAGGGtTGGGGtTGGGGCACAC-5′	Mutated IGFBP-5
BP5-PDREm3	5′-CCTCTCCCaACCCCCACCCCGTGTG-3′ 3′-GGTGTGGGtTGGGGGTGGGGCACAC-5′	Mutated IGFBP-5
BP5-PDREm4	5′-CCTCTCCCCACCCCaACCCCGTGTG-3′ 3′-GGTGTGGGGTGGGGtTGGGGCACAC-3′	Mutated IGFBP-5
c-fos RCE (Robbin et al., 1990)	5′-CGCGCCACCCCTCTGGCGCCACCGTG-3′ 3′-GCGCGGTGGGGAGACCGCGGTGGCAG-5′	Human c-fos
TGF-β RCE (Kim et al., 1991)	5′-CGCCCCCGGCCCCACCCCAGGAAG-3′ 3′-GCGGGGGCCGGGGTGGGGTCCTTC-5′	Human TGF-β1
MNF (Bassel-Duby et al., 1992)	5′-ACGCACAACCACCCCACCCCCTGTG-3′ 3′-TGCGTGTTGGTGGGGTGGGGGACAC-5′	Human myoglobin
AP-2 (Williams, et al. 1988)	5′-GATCGAACTGACCGCCCGCGGCCCGT-3′ 3′-CTAGCTTGACTGGCGGGCGCCGGGCA-5′	Metallothionein -IIA
Sp1 (Brigg, et al. 1986)	5′-AATCGATCGGGGCGGGGCGAGC-3′ 3′-TTAGCTAGCCCCGCCCCGCTCG-5′	Simian Virus 40 early promoter

Underline indicates CACCC sequence. Lower case letter indicates mutated nucleotide.

Figure 18. EMSA of BP5-PDRE binding to U2 cell nuclear factors. All oligodeoxynucleotide sequences are shown in Table 1. **A)** ^{32}P-labeled BP5-PDRE was incubated with 3 µg of U2 cell nuclear extract as described in Methods in the absence (lane b) or presence of a 50 fold molar excess of unlabeled BP5-PDRE (lane c), BP5-PDREm1 mutant with six altered nucleotides in the CACCC boxes (lane d), consensus AP-2 (lane e) and Sp1 (lane f) sequences, and RCE sequences from TGFß and fos promoters (lane g and h, respectively). Lane a shows the ^{32}P-labeled BP5-PDRE in the absence of nuclear extract. **B)** ^{32}P-labeled BP5-PDRE was incubated with 3 µg of U2 nuclear extract in the absence (lane b) or the presence of 25, 50, and 100 fold molar excess of unlabeled BP5-PDRE (lane c, d, and e, respectively), 25, 50, and 100 fold molar excess of CACCC core sequence containing myocyte nuclear factor (MNF) binding sequence from the human myoglobin promoter (lane f, g, and h, respectively), or 50 fold molar excess of unlabelled BP5-PDRE CACCC box mutants. In BP5-PDREm2 (lane i), both CACCC sequences are mutated to AACCC, in BP5-PDREm3 only the distal CACCC sequence is mutated to AACCC and in BP5-PDREm4 (lane k) only the proximal CACCC sequence is mutated.

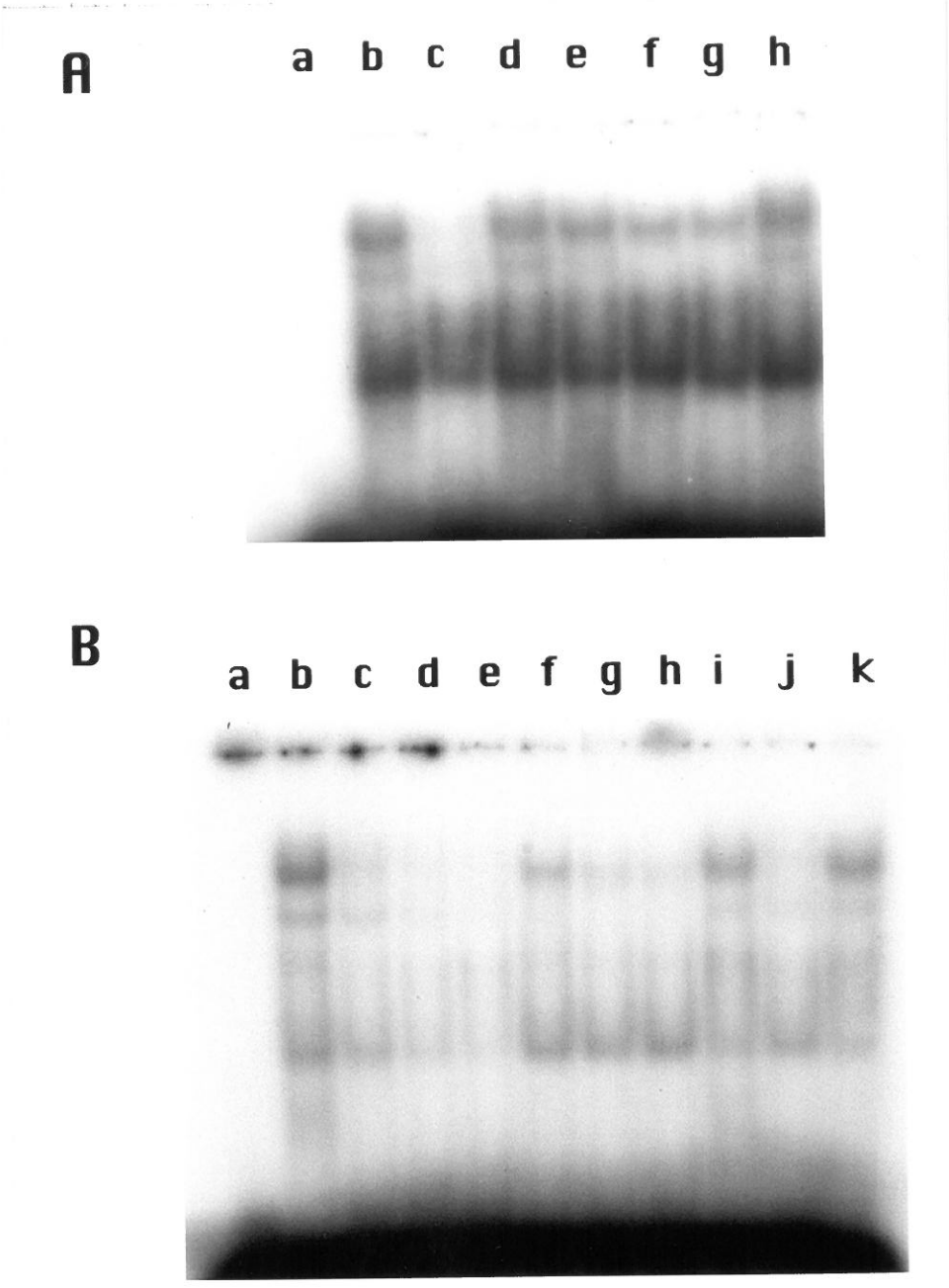

A
a b c d e f g h
B
a b c d e f g h i j k

Figure 19. BP5-PDRE binding factor(s) is an MNF-related transcription factor(s). Cold competition EMSA assay using wild type BP5-PDRE, MNF, and AP-2 oligodeoxynucleotides as competitors. ^{32}P-labeled BP5-PDRE was incubated with 3 µg of U2 nuclear extract in the absence (lane b) or the presence of 25 and 50 fold molar excess of unlabeled BP5-PDRE (lane c and d), 25 and 50 fold molar excess of MNF binding sequence (lane e and f), or 50 and 100 fold molar excess of AP-2 consensus sequence (lane g and h) prior to analysis by EMSA.

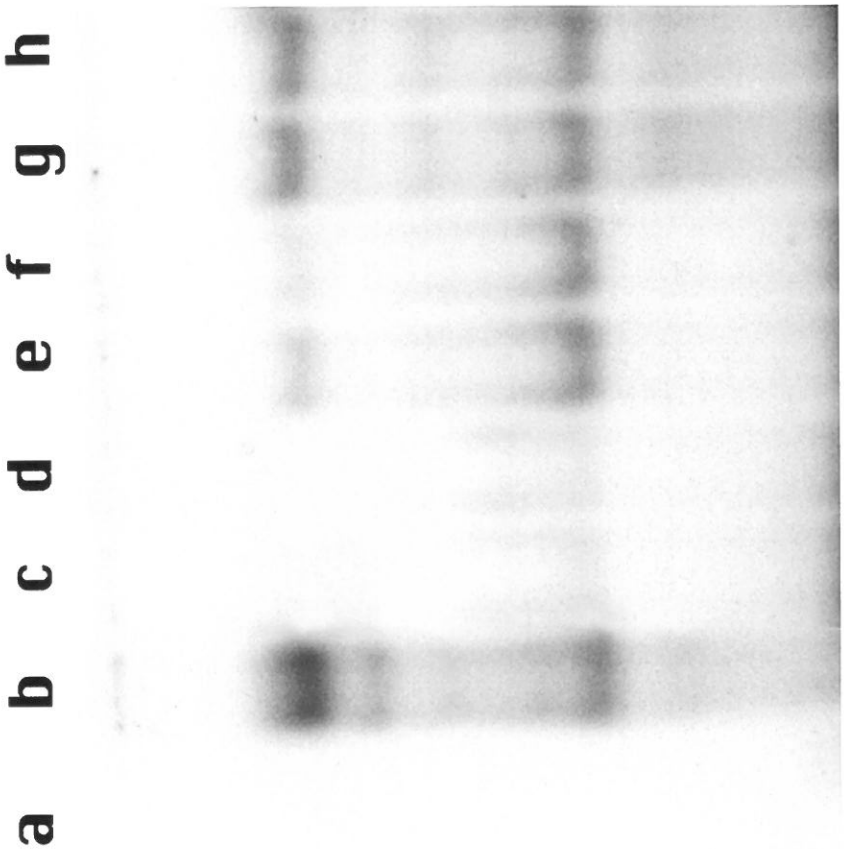

a b c d e f g h

Figure 20. PR-A interaction with nuclear BP5-PDRE binding protein(s). A) ^{32}P-labeled consensus MMTV-PRE was incubated without proteins (lane a) or with 1 µg of U2 cell nuclear extract (lane b), 5 fmol of purified PR-A (lane c) or a combination of nuclear extract and PR-A (lane d) prior to analysis by EMSA. Lane e is the same as lane d except 1 µg of PR antibody, AB52, was added as described in Methods. **B)** ^{32}P-labeled BP5-PDRE was incubated with 5 µg of U2 cell nuclear extract (lane a) in the presence of increasing amounts of PR-A (lane b-f). Numbers above each lane indicate fmol of PR-A. PR antibody, AB52, was added to the mixture run in lane f.

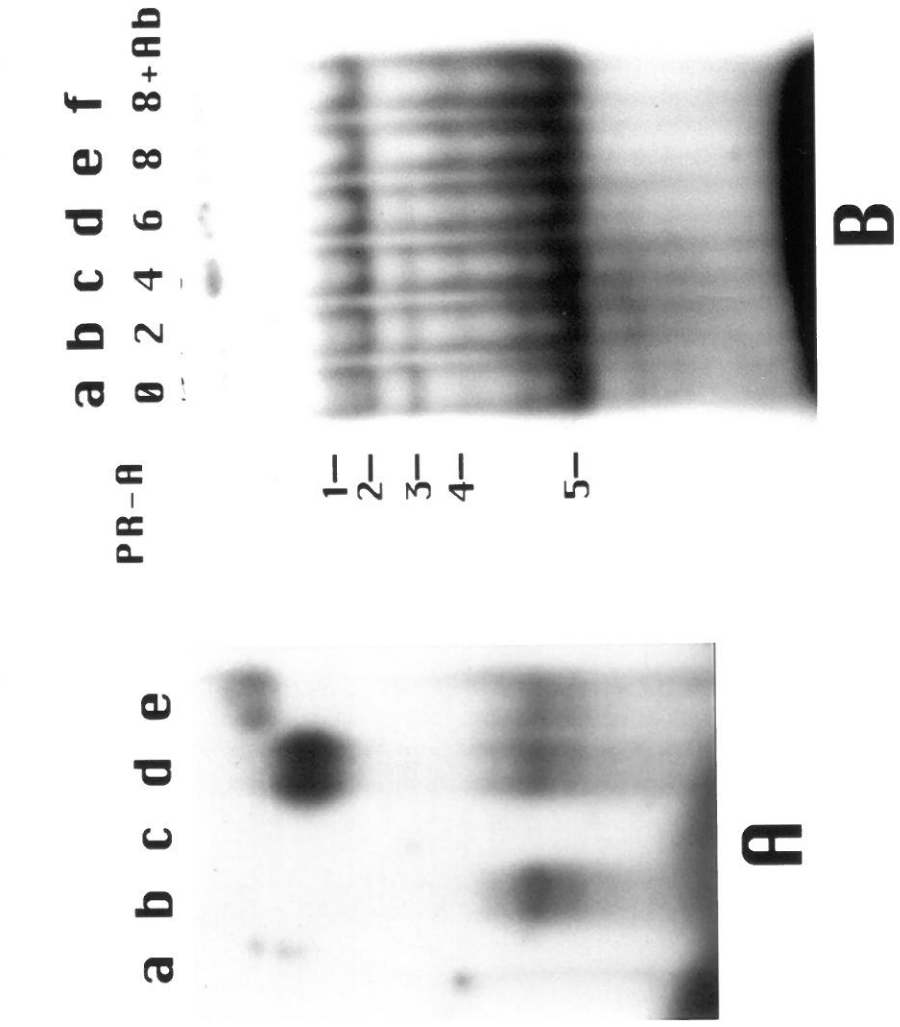
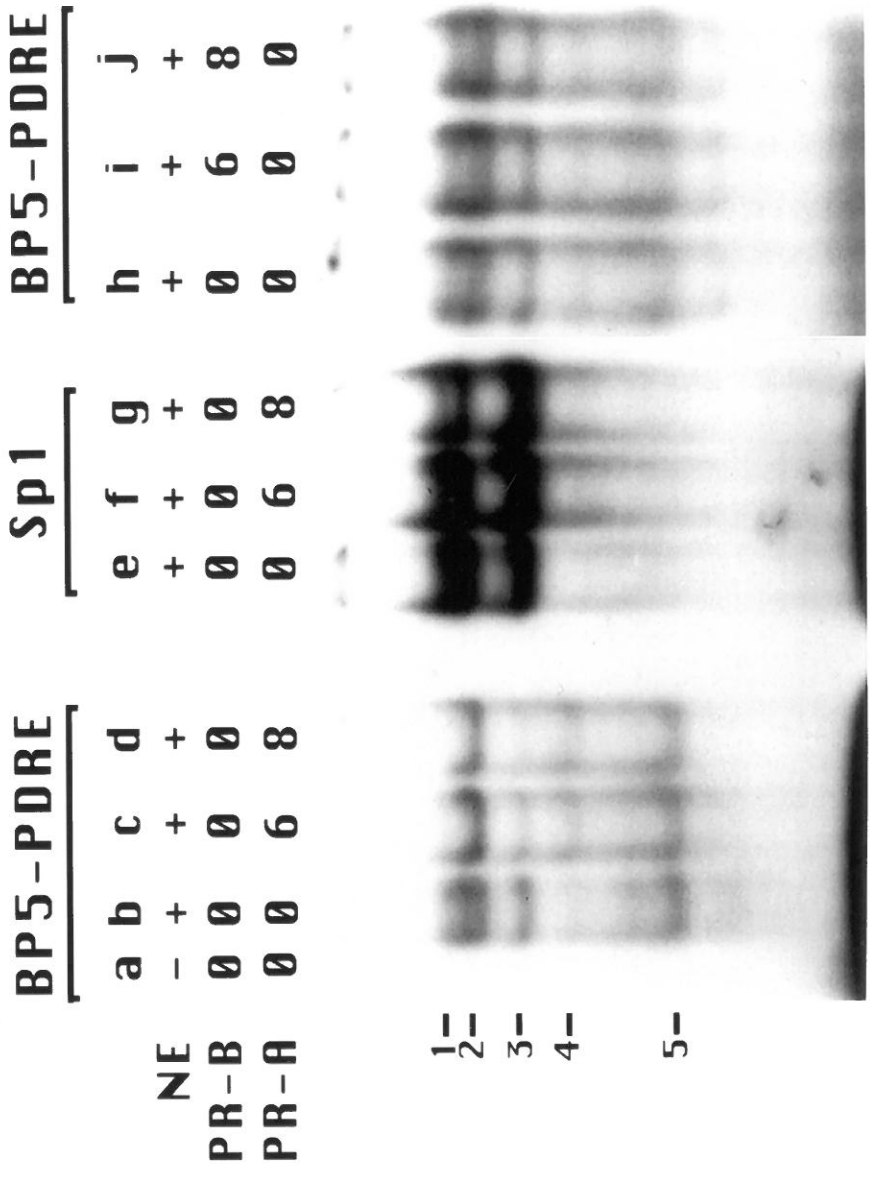

a b c d e f
PR-A ø 2 4 6 8 8+Ab
1—
2—
3—
4—
5—
B
a b c d e
A

Figure 21. The interaction between PR-A and BP5-PDRE binding protein(s) is specific. ^{32}P-labeled BP5-PDRE or Sp1 oligonucleotide (indicated above lanes) were incubated with 5 μg of U2 cell nuclear extract (NE), with or without the addition of purified PR-A (lane a-g) or PR-B (lane h-j) prior to analysis by EMSA. Numbers above each lane indicate fmol of purified PR-A or PR-B. PR-A altered the gel shift pattern of BP5-PDRE but did not affect the gel shift pattern of Sp1 binding sequence. PR-B did not affect the BP5-PDRE gel shift pattern.


BP5-PDRE
Sp1
BP5-PDRE
a b c d e f g h i j
NE - + + + + + + + + +
PR-B 0 0 0 0 0 0 0 0 6 8
PR-A 0 0 6 8 0 6 8 0 0 0
1
2
3
4
5

CHAPTER THREE

IV. DISCUSSION

A. Effect of PG on IGF System Regulation of Bone Metabolic Processes

Clinical studies suggest that treatment of postmenopausal women with PG or related progestagens reduces bone loss, either given alone or in combination with estrogen (Abdalla et al., 1985; Horowitz et al., 1993; Prior, 1990). However, the mechanisms underlying PG protective effects on bone mass are unclear. When the combination of progestagens and estrogen was given to postmenopausal women, there was an increased serum alkaline phosphatase and osteocalcin, markers of bone formation, more than effects of estrogen alone (Christiansen et al., 1985). In addition, Grey et al. (1996) reported that combined estrogen and medroxyprogesterone therapy in osteoporotic post-menopausal women resulted in a 65% greater increment in spinal bone mineral density compared to unopposed estrogen treatment. Since it has been recently demonstrated that the primary effect of estrogen is to inhibit bone resorption with very minimal effect on bone formation (Jilka et al., 1992), the bone formation effect of the combination treatment could, in part, be contributed to progestagens. Consistent with this observation, it has been demonstrated in animal studies progestagen treatment alone resulted in decreasing bone resorption and increasing bone formation as evidenced by decreased a marker of bone resorption, urinary hydroxy proline, and increased alkaline phosphatase and osteocalcin. (Barengolts et al., 1990; Karambolava et al., 1986; Snow and Anderson, 1985).

phosphatase and osteocalcin. (Barengolts et al., 1990; Karambolava et al., 1986; Snow and Anderson, 1985).

In vitro studies have sought to determine mechanisms underlying PG effects on bone formation. PG increased human osteoblast proliferation and differentiation (Lau et al., 1994; Lempert et al., 1992; Tremollieres et al., 1992; Wei et al., 1993) transiently increased IGF-II, type I , and type 2 IGF receptor mRNA levels in normal human bone cells and increased the sensitivity of the cells to IGF-II stimulation of proliferation (Lempert et al., 1992). In addition to increasing IGF-II and IGF receptor expression, PG also alters the IGFBP expression pattern to further increase IGF activities in the osteoblast microenvironment by decreasing expression of an inhibitory IGFBP, IGFBP-4, and increasing expression of the stimulatory IGFBP, IGFBP-5.

Since PG increases IGF-II, and IGF-II increases IGFBP-5 production, it is possible that PG increases IGFBP-5 expression indirectly by increasing IGF-II production. However, when PG was added concomitantly with a protein synthesis inhibitor, cycloheximide, we found that new protein synthesis was not required for PG induction of IGFBP-5 mRNA levels. This result suggests that PG directly increases IGFBP-5 mRNA levels by a mechanism that does not require increased IGF-II expression and did not involve the increase in IGF-II expression. Furthermore, result of nuclear run-on analysis demonstrate that PG directly increases IGFBP-5 gene transcription as early as 1 h.

Since IGFBP-5, when added to osteoblast cell cultures, increased basal and IGF stimulated proliferation (Andress and Birnbaum, 1991; Bautista et al., 1991; Kiefer et al., 1991; Mohan et al., 1995), it is very likely that PG stimulation of human osteoblast proliferation *in vitro* and the positive effects of progestagens on bone formation *in vivo* may be mediated, in part, by PG induction of IGFBP-5 production. The increase in IGFBP-5 mRNA levels and subsequent increase in secreted IGFBP-5 protein in the osteoblast microenvironment could result in an increase in IGF activities and increase in osteoblast proliferation.

B. Comparison of the hIGFBP-5 Promoter to Other IGFBP Promoters

The human IGFBP-5 proximal promoter contained both CAAT and TATA boxes, common elements of basal proximal promoters of many genes. Of six known human IGFBP promoters, the IGFBP-1 promoter is most like the hIGFBP-5 promoter in that it also contains a CAAT box and a TATA box, at similar positions relative to the start of transcription (Brinkman et al., 1988; Cubbage et al., 1989). In contrast, the human IGFBP-3 (Cubbage et al., 1990) and rat IGFBP-4 (Gao et al., 1993) promoters contain a TATA box between 37 to 40 bp from the transcription initiation site but do not contain a CAAT box. No TATA or CAAT boxes were present in human or rat IGFBP-2 proximal gene promoter regions (Binkert et al., 1992; Brown and Rechler, 1990). In contrast to human IGFBP-1, -2, and -3, the human IGFBP-5 promoter gene does not contain a CpG island.

More distal to the CAAT and TATA boxes, the human IGFBP-5 promoter sequence contains several possible response elements that may mediate PG effects on transcription. The consensus sequence for GRE/PRE is composed of two hexameric half-site arranged as a palindrome with a 3 base pair spacer (GGTACANNNTGTTCT) (Lieberman et al., 1993). None of the IGFBP promoters contain the perfect consensus GRE/PRE sequence. The IGFBP-1 promoter is the only IGFBP promoter which contains functional imperfect or nonconsensus GRE/PRE. The rat IGFBP-1 promoter contains a single 15 bp GRE/PRE sequence (TGAACACGGGGATCC) (Goswami et al., 1994), and the human IGFBP-1 also contains a similar 15 bp GRE/PRE sequence (TGAACAAACAGGTCC) (Suwanichkul et al., 1994). Both GRE/PRE sequences bind purified GR in DNA footprinting experiments and mutation of these GRE/PRE sequences disrupts the effect of glucocorticoid induced IGFBP-1 gene transcription (Goswami et al., 1994; Suwanichkul et al., 1994). Nine of the 12 nucleotides that comprise the 5'- and 3'- half-sites of the rat IGFBP-1 GRE/PRE sequence (position -7 to -5 and position +2 to +7) were identical to the human IGFBP-5 sequences identified as PRE-4 and PRE-5 (Figure 10). In contrast to the IGFBP-1 GRE/PRE, as shown by deletion analysis of the hIGFBP-5 promoter, these putative PREs did not seem to function and were not required for PG induction of hIGFBP-5 gene transcription. Instead, the 5'-flanking region between -162 to -124 which contained CACCC sequences was required for the induction.

Comparison of the human IGFBP-5 promoter CACCC sequence in -162 to -124 to the corresponding reported rat (Zhu et al., 1993) and mouse IGFBP-5 sequences (Kou

et al., 1994) (Figure 10) showed that this region was very well conserved among species, suggesting that it may contain important elements required for transcriptional regulation of the IGFBP-5 gene. Thus, it is possible that the results obtained from the studies of these CACCC sequences in the hIGFBP-5 gene promoter will also apply to mouse and rat IGFBP-5 promoters.

C. CACCC Sequences in the hIGFBP-5 Promoter and BP5-PDRE Binding Protein(s)

From deletion and mutation analysis, the sequence responsible for PG induction of hIGFBP-5 promoter activity was mapped to the CACCC box sequences between -124 and -162 and specifically to the proximal CACCC box. None of the putative PRE half-sites in the hIGFBP-5 proximal promoter is required for the induction. CACCC boxes have been identified in the proximal promoters of other genes such as mouse and human myoglobin (Bassel-Duby et al., 1992; Blanchelot et al., 1992), muscle creatine kinase (Horlick and Benfield, 1989), slow-cardiac troponin C (Parmacek et al., 1992), tryptophan oxygenase (Schule et al., 1988), and erythroid ß-globin (Weller et al., 1984). Although CACCC boxes are present in several genes, the precise function of the CACCC box sequences is still unknown.

A mechanism has been previously proposed for functional interaction between a CACCC box and GRE/PRE in transactivation of a promoter by glucocorticoid. The presence of a CACCC-box 15-39 bps from a consensus GRE/PRE in the tryptophan

oxygenase promoter enhanced GR/PR binding to the GRE/PRE and stimulated gene transcription (Schule et al., 1988). Mutation of the CACCC box abolished this synergism (Schule et al., 1988). Because the hIGFBP-5 promoter CACCC box is in close proximity to imperfect GRE/PRE sequences, it is possible that transacting factors binding to the CACCC box interacted with PR and synergized with PR to induce hIGFBP-5 gene transcription. However, deletion analysis of the hIGFBP-5 promoter showed that the same fold induction of the hIGFBP-5 promoter was obtained when cells were transfected with either pCAT252, which contained PRE-4, PRE-5 and CACCC boxes, or pCAT162 which contained only the CACCC boxes. Thus, there is no evidence supporting an interactive mechanism between the putative PREs and CACCC box in the hIGFBP-5 promoter.

Since deletion and mutation analysis showed the proximal CACCC box in the hIGFBP-5 promoter is required for PG transactivation, experiments were done to determine what type of transcription factor proteins might bind to the CACCC box. EMSA with U2 cell nuclear extract and labeled BP5-PDRE demonstrated several protein-DNA complexes. Several CACCC sequences previously identified in other gene promoters were compared to the hIGFBP-5 CACCC box by EMSA. Since a CACCC box sequence containing retinoblastoma control element (RCE) motif has been identified as an important sequence conferring RB-mediated regulation of gene expression (Kim et al., 1992; Kim et al., 1991; Kim et al., 1992; Pietenpol et al., 1991; Robbin et al., 1990; Yu et al., 1992), it is possible that BP5-PDRE binding proteins could be RCE binding

proteins. To assess whether the factor(s) binding to the CACCC sequence (BP5-PDRE) might be similar to RCE binding factors, two RCE oligodeoxynucleotides with sequences similar to but distinct from CACCC sequences (Table 1) were used in cold-competition experiments. None of the RCE oligodeoxynucleotides tested was as effective as the wild type BP5-PDRE in competing for the trans-acting factors which bound to BP5-PDRE. Therefore, it is unlikely that the BP5-PDRE binding factor is an RCE binding protein.

In addition to RCE binding factors, CACCC sequences may be bound by members of the Sp1 family (Sp1, Sp2 and Sp3) or AP-2, ubiquitous DNA binding factors that activate transcription (Faisst and Meyer, 1992; Kim et al., 1992; Kingsley and Winoto, 1992). Recently, Kinsley and Winoto (1992) demonstrated that members of the Sp1 family, Sp2 and Sp3, bind strongly to the Sp1 binding site. Therefore, to determine whether BP5-PDRE binding protein was AP-2 or a member of the Sp1 family, consensus AP-2 and Sp1 DNA sequences were used in cold-competition EMSA. Although Sp1 oligodeoxynucleotide partially competed for the binding with bands 1 and 3 and AP-2 oligodeoxynucleotide partially competed for binding with band 3 (Figure 18), both consensus oligodeoxynucleotides still were less effective as wild-type BP5-PDRE oligodeoxynucleotide in partially competing for binding with BP5-PDRE binding protein(s). Thus, the results suggest that BP5-PDRE binding protein(s) were not AP-2 or members of the Sp1 family.

The sequence CCCACCCC which confers PG responsiveness in the hIGFBP-5 promoter was identical to the binding sequence of myocyte nuclear factor (MNF) in

human myoglobin promoter (Bassel-Duby et al., 1994). MNF is a novel winged-helix (HNF-3/fork head) transcription factor which interacts with other factors such as myocyte-specific enhancer binding factor 2 (MEF-2) to confer muscle-specific transcription to the human myoglobin promoter (Bassel-Duby et al., 1992). MNF expression is increased during myocyte differentiation (Bassel-Duby et al., 1994). To determine whether the BP5-PDRE binding protein could be MNF, MNF binding site oligodeoxynucleotides were used in cold-competition EMSA. Unlabeled wild type BP5-PDRE oligodeoxynucleotide sequence as low as 25 fold molar excess completely competed for the proteins which bound to the labeled BP5-PDRE whereas the MNF binding-site oligodeoxynucleotide required up to 50 fold molar excess for effective competition. In contrast, the AP-2 consensus oligodeoxynucleotide sequence as high as 100 fold molar excess was not effective in competing for binding (Figure 19). Thus, among all the consensus binding sites tested, the MNF binding site oligodeoxynucleotide was the best competitor for binding to the BP5-PDRE binding protein(s), suggesting that BP5-PDRE binding proteins may be most closely related to MNF.

Interestingly, IGFBP-5 is abundantly expressed in muscle cells in the developing embryo and during terminal differentiation of several myogenic cell lines (James et al., 1993). By transfecting deletion promoter-reporter constructs of the mouse IGFBP-5 promoter into C2I myoblast cells, Rotwein et al. (1995) demonstrated that the proximal 156-bp fragment, which containsthe same CACCC box sequences as in hIGFBP-5

promoter, is responsible for the differentiation dependent rise in IGFBP-5 gene transcription. Based on sequence homology and the importance of this sequence for the expression of IGFBP-5 in muscle cells during differentiation, Rotwein et al. (1995) suggested that MNF bound to these CACCC boxes. However, the results from our cold competition studies suggest that BP5-PDRE binding proteins or CACCC binding proteins were not identical to MNF since wild type BP5-PDRE oligodeoxynucleotide competed better than MNF oligodeoxynucleotide for binding. Although MNF is unlikely to be the BP5-PDRE binding protein(s), it is possible that BP5-PDRE binding proteins could be MNF-related transcription factors since both IGFBP-5 and MNF expression were induced during myocyte differentiation and among all the CACCC sequences tested the MNF-oligodeoxynucleotide competed most effectively for binding to the labeled BP5-PDRE oligodeoxynucleotide.

Since both proximal and distal CACCC boxes of the BP5-PDRE sequences were very similar, it is interesting to speculate that both CACCC boxes could interact with BP5-PDRE binding proteins. However, according to mutation analysis, only the proximal CACCC box was required for the binding of BP5-PDRE binding proteins, and for transactivation of the promoter by PG. This may be due to differences in the proximal flanking region in each box which could influence the binding of BP5-PDRE binding proteins. Bassel-Duby et al. (1992) demonstrated that mutation of the MNF binding site flanking sequence had a minimal effect on promoter activity. This suggested that MNF site flanking sequences had very little influence on the binding of MNF. Since the results

from cold competition assays suggested that BP5-PDRE binding factors could be MNF or a related transcription factor, it is possible to speculate that the flanking region of the CACCC box in hIGFBP-5 gene has a minimal effect on the binding of the BP5-PDRE binding protein to the CACCC box. Alternatively, mutation of each CACCC box could cause different changes in DNA secondary structure and influence the ability of proteins to bind to the BP5-PDRE binding protein. The mutation of the proximal CACCC box, which abolished the binding of BP5-PDRE binding proteins, may result in DNA secondary structures which are unfavorable for binding to the BP5-PDRE binding proteins.

D. Interaction of Steroid Hormone Receptors with Other Transcription Factors

Classically, ligand-activated steroid hormone receptors stimulate gene transcription through direct binding to consensus hormone responsive elements (HREs) (Rories and Spelberg, 1989). Unactivated steroid hormone receptors *in vivo* have either neutral or silencing activity in the absence of ligand. Upon binding to the hormone, the receptor becomes a positive regulator. In the absence of hormone, PR and GR have been shown to exist as 8-10 S complexes associated with heat-shock proteins (hsp90, hsp70, and hsp56) (Georgopoulus and Welch, 1993; Hendrick and Hartl, 1993; Kost et al., 1989). Upon binding ligand to the receptor, heat-shock proteins dissociate, receptors dimerize, and the actual receptor dimers bind to specific hormone response element (HRE) DNA sequences and transactivate target genes (Tsai and O'Malley, 1994). The

removal of the heat shock protein, however, is not sufficient by itself to convert a steroid receptor from hormone-dependency to one that is constitutively active (Bagchi et al., 1991). Using a hormone-dependent, cell free system and purified receptor, Bagchi et al. (1991) demonstrated that receptor binding to DNA and transactivation of a target gene, requires the binding of hormone to the receptor even in the absence of heat shock protein. One explanation for this hormone requirement for receptor activation is that a conformational change is induced in the receptor by the hormone. Supporting this conclusion, EMSA studies have shown that hormone binding alters the gel shift pattern of purified steroid receptor-DNA complexes (Beekman et al., 1993). Using protease digestion and monoclonal antibody mapping to detect ligand-dependent conformational changes, Allan et al. (1992) showed that PG induces a conformational change in PR, converting the entire hormone-binding domain to a very compact structure.

The results of our studies suggest that PR mediated induction of the hIGFBP-5 gene did not involve direct binding of PR-A to PRE. The region responsible for PG responsiveness was mapped to a CACCC box. It is possible that binding of PR-A to the CACCC box stimulated hIGFBP-5 gene transcription. In support of this possibility, recent studies suggest that steroid hormone receptors stimulate gene transcription by mechanisms which are independent of direct binding to consensus HREs (Kutoh et al., 1992; Miner and Yamamoto, 1991; Norris et al., 1995; Sukovich et al., 1994). The receptors could activate gene transcription through novel DNA sites that differ substantially from the consensus HREs (Norris et al., 1995; Schweers et al., 1990;

Sukovich et al., 1994). Using a genetic selection system in yeast, Norris et al.(1995) identified a new subclass of *Alu* family DNA repeats in the BRCA-1 gene which functions as an estrogen receptor-dependent enhancer and confers receptor-dependent estrogen responsiveness to a heterologous promoter. In addition, Sukovich et al. (1994) identified a TA-rich sequence and a CCAAT sequence which were crucial for induction of the brain creatine kinase (BCK) promoter by the estrogen receptor. However, direct binding of ER to the sequences is not required for transactivation. Using deletion and mutation analysis, Schweers et al. (1990) identified steroid dependent regulatory elements (SDREs) in the ovalbumin promoter which did not have sequence homology to any of the consensus HREs. The SDREs conferred hormone (estrogen, glucocorticoid, progesterone and dihydroxytestosterone) responsiveness to the ovalbumin gene but did not bind to partially purified progesterone receptor. Since direct binding of the receptor to nonconsensus HREs is not required, it is, therefore, possible that the conformational changes induced by hormone binding allow the activated receptors to interact with other factors which bind to the nonconsensus HREs to activate transcription.

Analysis of the hIGFBP-5 promoter suggests that purified PR-A does not bind to the functional CACCC box but interacts with proteins or protein complexes bound to the CACCC box. Therefore, it is likely that ligand activated PR-A stimulates hIGFBP-5 gene transcription indirectly via interaction with transcription factors or cofactors which bound to the CACCC box. Supporting this conclusion, multiple factors have been

described which interact with steroid hormone receptors in a ligand-dependent manner and modulate receptor activities. These factors can be grouped into three categories: (1) other nuclear transcription factors, (2) nuclear factors which are required for efficient DNA binding by steroid hormone receptors ,and (3) transactivating function intermediary factors (TIFs) which mediate the transcription activation function of the steroid hormone receptor to the basal transcription machinery. The three types of factors are discussed in the following paragraphs.

Steroid hormone receptors have been shown to bind to other nuclear transcription factors. Interactions of PR, GR and androgen receptor (AR) with AP-1 (Fos and Jun) components downregulate transcription of target genes (Jonat et al., 1990; Schule et al., 1990; Yang-Yen et al., 1990). Yang-Yen et al. (1990) showed that interaction of GR with AP-1 resulted in a complex that was unable to bind to DNA. Others have proposed that, depending on the relative composition of Jun and Fos, GR receptor activity can either be enhanced or inhibited (Shemshedini et al., 1991). These studies not only demonstrated that the binding of nuclear transcription factors to steroid hormone receptors modulate the receptor activities, they also showed the existence of the crosstalk between ligand-activated and phosphorylation-regulated pathways conferred by AP-1 at the nuclear level.

Nuclear factors which enhance the DNA binding of the steroid hormone receptors have been described. Two nonhistone chromosomal proteins that can bind to the spacer of palindromic EREs were shown to enhance ER binding (Fawell et al., 1990). In

addition, *in vitro* binding of highly purified human ER to an ERE requires the addition of single-stranded-DNA-binding proteins (Mukherjee and Chambon, 1990). Cavanaugh and Simmon Jr. (1990) showed that a 93-kDa protein increased binding of activated GR to nuclei and chromatin in an ATP-dependent manner. Edwards et al. (1989) showed that the maximum binding of PR to PREs *in vitro* was dependent upon both hormone and a nonreceptor nuclear factor (Edwards et al., 1989). Recently, Onate et al. (1994) demonstrated that the high-mobility-group chromatin protein (HMG-1) enhances the binding of both activated PR-A and PR-B to an MMTV-PRE by inducing a structural change in the target DNA.

In many studies overexpression of one receptor results in the squelching of its own or another receptor's activity (McDonnell et al., 1994; Meyer et al., 1989; Vegato et al., 1993; Wen et al., 1994). Furthermore, the functional activities of various transactivation domains of the ER vary in the context of different cell types (Shemshedini et al., 1992) which suggests that the target for various activation domains may be distinct. Since the basic transcription machinery exists in all genes, it is possible that factors other than general transcription factors, such as coactivators or transactivation function intermidiatory factors (TIFs), could play an important role in receptor activation. Several receptor associated TIFs have been identified. Shemshedini et al. (1992) have identified a TIF that eliminated the self-squelching effect of the estrogen receptor (ER) but had no influence on basal transcription. ER associated proteins ERAP160 (Halachmi et al., 1994), RIP160 and RIP80 (Cavailles et al., 1994) exhibit estradiol-dependent binding to

the ER. The binding of these ER associated proteins to the ER correlates with the ability of the ER to activate transcription *in vivo*. Recently, several authors used a yeast-based genetic system to demonstrate that several factors including SWI1, SWI2, SWI3, SIN3 and SPT6 (Baniahmad et al., 1995; Nawez et al., 1994; Yoshinaga et al., 1992) enhance the transcriptional activity of different steroid hormone receptors. Using a yeast two-hybrid system, Onate et al. (1995) identified a protein, steroid receptor coactivator-1 (SRC-1), which interacts with and enhances transactivation of steroid hormone receptors. Coexpression of SRC-1 reversed the ability of the estrogen receptor to squelch activation of PR (Onate et al., 1995). These coactivators were proposed to bridge receptors to the core transcription machinery which is consistent with the notion that multiple accessory factors are involved in receptor transactivation.

Several steroid hormone receptors including PR (Klein-Hitpass et al., 1990), ER (Elliston et al., 1992) and GR (Tsai et al., 1990) stabilize the formation of preinitiation complexes on DNA; however the precise mechanism is still unclear. In addition to interaction with TIF proteins, hormone receptors (COUP-TF, ER and PR) interact *in vitro* with the basal transcription factor, TFIIB (Ing et al., 1992). Since TFIIB has been reported to be a rate-limiting component of trancriptional activation (Lin and Green, 1991), Ing et al.(1992) proposed that binding of a steroid hormone receptor to its HRE recruits TFIIB into the initiation complex and stabilizes the complex. This stable complex allows subsequent binding of RNA polymerase II and other general transcription factors TFIIF, E, H and J to efficiently complete the formation of the preinitiation

complex and rapidly transcribe an activated gene. Consistent with these findings, VP-16, a viral transactivator, activates transcription by interacting with TFIIB (Roberts et al., 1993).

E. Proposed Mechanisms of PG Induction of hIGFBP-5 Gene Transcription

PG induction of hIGFBP-5 gene transcription is quite unique in that it is mediated through PR-A and not PR-B. In most PG inducible genes that have been examined, transcription is increased by binding of ligand-activated PR-B to a PRE within the gene promoter. To date, the only known example of a gene that is activated by PR-A and not PR-B is the chicken ovalbumin gene (Schweers et al., 1990), however, the detailed mechanism for this interaction was not determined. PR-A and PR-B are likely to mediate transcriptional regulation through different mechanisms because hPR-A in many cases functions as a dominant negative regulator and does not bind directly to an HRE (Tora et al., 1988; Vegato et al., 1993). PR-A has been proposed to act by binding in a nonproductive manner to a limiting cofactor protein that is required for activation of other steroid hormone receptors (Tung et al., 1993; Vegato et al., 1993). However, in contrast to other systems, we found that PR-A functioned as an activator of hIGFBP-5 gene transcription.

Interestingly, PR-A and PR-B differ only in the length of their N-terminus but yet they have different functional properties. It is possible that loss of 164 amino acid residues at the N-terminus of PR-A could result in a different folding structure compared

to PR-B. In addition, Beck et al demonstrated that both isoforms exhibit significant amounts of phosphorylation and undergo an increase in phosphorylation upon hormone stimulation (Beck et al., 1992). PR-A has fewer phosphorylation sites compared to PR-B (personal commumication, Dean P. Edwards). Both different folding and changes in phosphorylation sites could cause PR-A to function differently from PR-B by interacting with different sets of proteins or transcription factors.

There are at least three possible mechanisms by which PR-A could mediate transactivation of the hIGFBP-5 gene. Regarding the classical mechanism of PG induction of gene transcription, activated PR dimerize, bind to the consensus PRE, and activate gene transcription. However, PREs half-sites in the hIGFBP-5 gene 5'-flanking region from -753 and +23 were not functional. Although PR does not bind effectively to PRE half sites, clusters of GRE/PRE half-site have been shown to bind to the receptor and mediate ligand dependent transactivation through a synergistic interaction between the multiple half-sites (Bailly et al., 1986; Beato et al., 1989; Lieberman et al., 1993). As shown in Figure 16, deletion of all PRE half-sites present in the hIGFBP-5 5'-flanking region in pCAT162 conferred PG responsiveness. According to the data presented, the induction of the hIGFBP-5 gene by PG did not involve a PRE or PRE half-site; therefore, it is unlikely that the transactivation follows a classical mechanism.

Since no PRE or PRE half-site is involved and a CACCC sequence is implicated in PG induction of hIGFBP-5 gene transcription, it is possible that the CACCC sequence binds to a negative regulator(s) or transcriptional repressor(s) which suppresse gene

transcription. In the presence of PG, the activated PR interacts with and removs the repressor(s) from the CACCC sequence, causing an increase in hIGFBP-5 gene transcription. Therefore, deletion of the CACCC box abolishing binding of CACCC binding proteins should increase gene transcription. However, deletion or mutation of both CACCC boxes did not cause any changes in basal promoter activity. Thus, the data argues against the possibility that a transcriptional repressor(s) binds to the functional CACCC sequences.

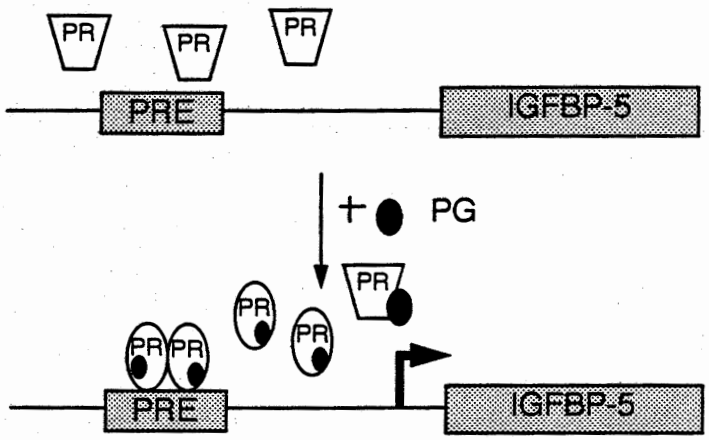
EMSA analysis shows there are at least five distinct protein-DNA complexes based on size, suggesting that the CACCC boxes interacted with proteins of different sizes or with different protein complexes. Although it remains to be determined whether CACCC box binds to individual proteins or protein complexes, there is considerable evidence supporting the notion that protein complexes bind to CACCC boxes. Several transcription factors such as the Sp1 family, AP-2, RCE-binding proteins, and MNF have been shown to bind to CACCC sequences (Bassel-Duby et al., 1992; Faisst and Meyer, 1992; Kim et al., 1992). However, the activity of the most CACCC binding proteins is regulated by binding of other transcription factors (Bassel-Duby et al., 1992; Kim et al., 1992). In the case of MNF which could be the BP5-PDRE binding protein or related to it, Bassel-Duby et al suggested that MNF is likely to require protein-protein interactions with other heterologous transcription factors such as MEF-2, which does not bind to the MNF binding sequence, for efficient transactivation in muscle cells (Bassel-Duby et al., 1992). Thus, it is very likely that BP5-PDRE binding proteins bind other transcription

factors which modulated their activities. In support of this conclusion, a CACCC box is required for efficient and accurate gene transcription in several genes (Bassel-Duby et al., 1992; Direks et al., 1983). Thus, proteins interacting with CACCC binding proteins may influence the efficiency of gene transcription.

As shown in Figure 20, PR-A caused the disappearance of at least two DNA-protein complexes and the appearance of at least one DNA-protein complex, suggesting that PR-A interacts with the CACCC or BP5-PDRE binding protein complex. In addition, direct binding of purified PR-A to labeled BP5-PDRE was not observed, suggesting that PR-A interacts with BP5-PDRE binding protein complexes rather than with BP5-PDRE DNA sequences. Several recent reports indicate that PR-A, in particular, interacts with limiting cofactors required for activation of other steroid hormone receptors (Tung et al., 1993; Vegato et al., 1993; Wen et al., 1994).

Evidence from our studies and others supports the hypothesis that BP5-PDRE binds to protein complexes which consist of CACCC binding protein(s) and other transcription factors. These factors may or may not bind directly to the CACCC box or flanking sequences. However, in the presence of ligand-activated PR-A, PR-A interacts with components of the BP5-PDRE binding protein complexes, removes the factors critical to the complex, and allowes other transcription activators to bind to the complexes and activate hIGFBP-5 gene transcription. A schematic representation of this model is shown in Figure 22.

Figure 22. Proposed novel molecular mechanism of PG action. A) Classical mechanism of PG action. Upon binding to PG, PRs undergo transformation and become activated PRs. Activated PRs (oval shape) then homodimerize, bind to PRE and activate gene transcription. **B)** Proposed novel molecular mechanism of PG action. In basal conditions before PG activation, transcription factors (hexagonal shape) bind to CACCC binding proteins and prevent transcription activators (pentagonal shape) from binding to CACCC binding proteins. Upon activation, activated PRs interact and form complexes with the inhibitory transcription factors and allow transcription activators to bind to CACCC binding proteins and activate gene transcription. In contrast to the classical model, activated PRs do not bind directly to the DNA elements, the CACCC boxes.


PR
PR
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+
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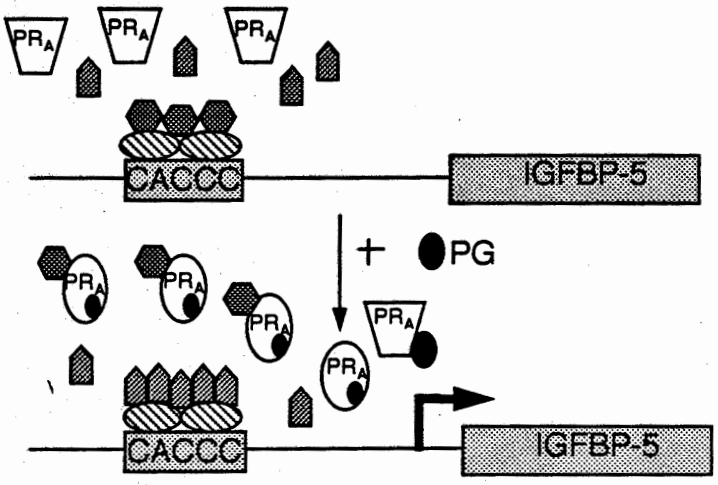
PRE
IGFBP-5

A


PRA
PRA
PRA
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IGFBP-5
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B

F. Conclusion

The studies presented here demonstrate that PG-induces an increase of steady state hIGFBP-5 mRNA primarily through a transcriptional mechanism. The significance of this study is that the element responsible for PG responsiveness was mapped to a CACCC sequence instead of a classical PRE. In contrast to our current understanding of PR activated gene transcription which involves the direct binding of PR to a PRE, our results suggested a novel mechanism of PG mediated transcriptional regulation. Direct DNA binding of a ligand-activated PR to the BP5-PDRE did not occur. Instead, activated PR-A interacts with BP5-PDRE binding proteins. To our knowledge, this is the first report of a mammalian gene in which transcription is activated by PR-A and not PR-B. The finding that PR-A, and not PR-B, interacts with other transcription factors or cofactors which bind to BP5-PDRE may help explain differences in the promoter and cell type specificity of PR-A and PR-B mediated gene transcription.

G. Future Directions

Although PR-A is structurally very similar to PR-B (PR-A lacks 164 amino acids at the N-terminus), the two receptor isoforms function differently. Studies defining the role of the first 164 amino acid region in the functional differences of the two PR isoforms will be informative. As mentioned earlier, there are at least two possible differences in the PR-A which might cause PR-A to interact with different sets of proteins: (1) the tertiary structure of PR-A may be different from PR-B, and (2) there are

fewer phosphorylation sites in PR-A. Since it is technically more feasible to test the second possibility, studies should be carried out to test this possibility. PR-B mutants will be used in which all phosphorylation sites within first 164 amino acids of the N-terminus are mutated. The mutated PR-B expression vector will be cotransfected with pCAT162 into U2 cells. The transfected cells will then be treated with PG for 24 h and CAT activity will be assayed. pCAT162 will be used since this construct contains the minimal promoter required for PG responsiveness. If mutant PR-B can activate hIGFBP-5 gene transcription but wild type PR-B cannot, then it is likely that the phosphorylation sites in PR-B prevent the receptor from interacting with appropriate proteins required to activate hIGFBP-5 gene transcription. If mutant PR-B fails to activate hIGFBP-5 gene transcription, then it is more likely that differences in the tertiary structure of PR-B and PR-A account for PR-A activating hIGFBP-5 gene transcription. A series of N-terminal deletions of PR-B could be made to progressively remove parts of the PR-B N-terminal sequences.

We have established that BP5-PDRE binding proteins bind to the CACCC boxes; however, further studies should be carried out to determine the significance of the flanking region of the functional CACCC boxes. First one should determine which nucleotides mediate BP5-PDRE binding using DNA footprinting analysis. This analysis will provide a picture of the nucleotides required for binding of BP5-PDRE binding proteins. Once the region required for binding is established, mutation analysis of the

CACCC box flanking region or the binding region will be carried out to verify the findings from DNA footprinting analysis.

Based on results obtained from this study, we propose that PR-A activates hIGFBP-5 gene transcription via a novel mechanism by which ligand-activated PR-A interacts with other transcription factors or cofactors in the BP5-PDRE protein complexes allowing a transcription activator to bind to the complexes and activate hIGFBP-5 gene transcription. It remains to be established whether BP5-PDRE DNA sequences interact with individual proteins or protein complexes. One can use preparative scale EMSA to determine how many proteins are in one DNA-protein complex by cutting the band representing each DNA-protein complex and analyzing the composition of the protein complex on a denaturing protein gel.

One of the most compelling studies which should be carried out is to determine the identity of the BP5-PDRE binding factors which interact with PR-A. Since PR-A acts as a dominant negative repressor of several steroid hormone receptors, several investigators have proposed that PR-A interacts with limiting factor(s) required for the activation of steroid hormone receptors (Tung et al., 1993; Vegato et al., 1993; Wen et al., 1994). To date only one cofactor has been demonstrated to interact with hPR. Using the yeast two hybrid system with the hPR ligand-binding domain as the bait, Onate et al. (1995) isolated a protein called steroid receptor coactivator-1 (SRC-1) which interacts with hPR. Coexpression of SRC-1 with either PR, GR, ER, TR, or RXR enhances the

activity of all of the receptors tested. It would be interesting to test whether SRC-1 could interact with BP5-PDRE binding proteins or PR-A by using EMSA.

The yeast two hybrid system can be used to isolate protiens in U2 cells which interact with hPR-A. One can use the yeast two hybrid system with hPR-A cloned into a vector containing the GAL-4 DNA binding domain and a library of cDNAs encoding proteins expressed in U2 cells cloned into a vector containing GAL-4 activation domain. The interaction between the factors and hPR-A *in vivo* will reconstitute the GAL4 transcription activator function and will result in transcription of the reporter gene.

After the cDNA encoding factors interacting with PR-A have been cloned, further studies to identify factors which are involved in PG activation of hIGFBP-5 gene transcription should be carried out. Since we propose that PR-A removes factors which blocked the binding of transcriptional activators, overexpressing such factors which interacts with PR-A should abolish PR-A's ability to activate hIGFBP-5 gene transcription. Since PR-A has been proposed to function in a cell- and promoter- specific manner (Tora et al., 1988), it will be important to investigate the expression pattern of these factors in different cell types to gain more understandings of how PR-A functions in those cells.

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